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PRECIPITATION OF THE TOBACCO MOSAIC VIRUS COMPLEX AT ITS ISOELECTRIC POINT

by

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(Submitted for publication 8th February, 1936.)

INTRODUCTION.

The desirability of obtaining pure preparations of plant viruses in order to study their nature and properties has long been recognized. The present paper describes the precipitation of the virus of ordinary tobacco mosaic (Johnson's tobacco virus 1) from clarified samples of infective juice by adjusting the pH value to what appears to be the isoelectric point of the virus itself or of something in the nature of a virus-substrate complex.

Vinson and Petre (1929, 1931) and Petre (1935) have developed a method of purification for this virus based on fractional precipitation with basic and neutral lead acetate and subsequent elution with phosphate solutions. Precipitation of the virus by means of safranin and subsequent removal of the safranin with amyl alcohol (Vinson, 1927, 1932; Vinson and Petre, 1929) and other methods have also been used. In these cases the virus is undoubtedly precipitated, but since this is brought about by well-known protein precipitants it is very likely that the product contains material other than the virus. But in view of the fact that the virus behaves like a protein in so far as its precipitation from the juice of diseased plants is concerned, it occurred to the author that it might possess an isoelectric point, and that if this were so a simple and efficient method of separation and purification might be developed along these lines. Since no data bearing on this point appear in the literature some preliminary experiments were performed, and these showed that the virus was precipitated at pH values between 3 and 4, and that it was not precipitated at pH values of 2·5, 5, 6, and 7.

These precipitates when re-suspended in a buffer solution of pH 7 were indistinguishable, as far as the concentration of active virus units was concerned, from untreated aliquots. The results of quantitative work designed to determine the "isoelectric" point or range of the precipitate is described below. In this work virus "cultured" in tomato plants as well as in tobacco plants has been used. Although the precipitate appears to be a homogeneous one, the possibility that it

may be an adsorption complex cannot be overlooked, and for this reason the general term "virus-complex" will be used to describe it at present.

EXPERIMENTAL TECHNIQUE.

Source of Virus.

Plants of *Nicotiana tabacum* (var. Blue Pryor) were artificially infected with the virus, and were stored in an isolated compartment of an insect-proof glasshouse. About two months after inoculation the leaves were removed, crushed in a mortar, and the juice filtered through muslin. The crude juice was allowed to stand exposed to air (but protected with a cotton wool plug) for 24 hours, and was then centrifuged at about 1,500 g. for 1 to 2 hours. The clear transparent reddish-brown supernatant liquid so obtained was filtered through a No. 44 Whatman filter paper, and the filtrate used as such for precipitation studies. This filtrate is referred to throughout as "clarified juice" or simply as "juice".

Plants of *Lycopersicum esculentum* (var. Dwarf Champion) artificially inoculated with the same virus and stored in the same compartment, were treated in the same way to obtain a clarified suspension of the virus in the juice of tomato plants. The suspensions of tomato plant juice were never quite transparent, nor were they as stable as those obtained from tobacco plants.

Buffer Solutions.

Buffer solutions of various pH values were prepared from a stock composite buffer (phthalate-phosphate-borate) by the addition of the appropriate amounts of NaOH or HCl solutions. The final concentration of these buffer solutions was 0·04 M. and since the same stock solution was used to cover the whole pH range, any differential effects due to the buffering substances were eliminated.

Preparation of Virus Suspensions.

Aliquots (1 ml.) of the same stock infective juice were added to centrifuge tubes containing buffer solutions of various pH values (9 ml. each). After standing for about half an hour the tubes were centrifuged at about 1,500 g. for 20 minutes. Aliquots of the supernatant liquids were then used to make up suspensions in a buffer solution of pH 7 so that the final suspensions (inocula) contained the equivalent of one part of juice in 1,000 total volume, 1 part in 1,500, or 1 part in 2,000, depending on the estimated susceptibility of the test plants. In any one experiment the same dilution was used for all inocula.

Later, when the conditions for maximum precipitation of the virus complex from the clarified juice had been determined, a preparation of the complex was re-suspended in buffer solution at pH 7, and aliquots of this suspension were added to buffer solutions of various pH values. The percentage precipitation of virus at these pH values was then determined as before.

Estimation of pH Values.

The pH values of the supernatant liquids, after centrifuging, were determined by means of the hydrogen and quinhydrone electrodes. The values recorded thus represent the pH values of the media with which the precipitates were in equilibrium.

Estimation of Relative Virus Concentration.

Inocula prepared as above were compared against each other, and a common control inoculum by the primary lesion method using *Nicotiana glutinosa* plants trimmed to three or four leaves per plant. The procedure was essentially that previously described (Samuel, Best, and Bald (1935), Best and Samuel (1936)). For any experiment the plants were arranged in a square of 64, 36, or 16 plants, according as there were 8, 6, or 4 test systems. Latin squares had pre-

viously been planned for each set of leaves, and the test systems were inoculated by means of flat ground glass spatulas on to one half of each leaf according to plan, and the common control inoculum was inoculated on to the other half of every leaf.

Since, under the conditions and concentrations used in this work, the number of primary lesions is directly proportional to the number of active virus units in the inoculum,[1] the ratios given in the tables and graphs represent relative concentrations of active virus units correctly within the experimental error of the method. All inocula were identical with respect to the nature of the suspension medium, the electrolyte concentration, and pH value. Previous experiments had shown that the virus was most stable in solutions at pH about 7, so that in this work the control inocula were prepared in buffer solutions of this pH value. In any one experiment the test plants were raised at the same time under the same conditions, they were all transferred to the laboratory at the same time (24 hours before inoculation), and the conditions under which the lesions developed were the same for all.

EXPERIMENTAL RESULTS.

Preliminary experiments had shown that precipitation of the bulk of the virus complex took place at a pH value of about 3·4 when the concentration of juice was 1 in 25. It was also found that comparable results were obtained whether the juice concentration was 1/10, 1/25, or 1/50, and on this account all future precipitation tests were done with a juice concentration of 1 in 10. It was also found that the greater part of the virus complex was precipitated at pH 3·4 whether a composite buffer (·04 M or ·08 M) phthalate buffer (·04 M), acetic acid (·05 M), or acetic acid (·1 M) was used.

The "isoelectric point" and the extent of the precipitation seemed to be relatively independent of the composition and concentration of the buffer solution. Nevertheless in order to eliminate any differential effects which might result from the use of different buffering substances all solutions were made up from the stock composite phthalate-phosphate-borate buffer.

The Reversible Nature of the Precipitation.

It is necessary to distinguish between a possible inactivation of the virus and a precipitation without real inactivation. In previous work (Best and Samuel, 1936) no significant changes were observed in virus activity after 12 hours' contact with buffer solutions over the pH range 2 to 8. Any permanent inactivation must therefore be small, but in order to put the matter beyond doubt the virus complex was precipitated at or near its isoelectric point from an aliquot of the clarified juice. The precipitate so obtained was re-suspended in buffer solution at pH 7, equal in volume to that of the juice from which the precipitate was obtained. An aliquot of this suspension was used to make up an inoculum in a buffer at pH 7 to give an "equivalent juice concentration" of 1 in 1,000. The supernatant liquid corresponding to the above precipitate was used to prepare a second inoculum also in buffer solution at pH 7, and of an equivalent juice concentration of 1 in 1,000.

[1] Unpublished results of dilution experiments by the author.

At the same time a control inoculum was prepared by adding an aliquot of the same stock from which the virus had been precipitated to buffer solution at pH 7 to give a juice concentration of 1 in 1,000. These inocula were inoculated against each other on opposite halves of *N. glutinosa* leaves.

The lesion count showed that: (1) there was no significant difference between the concentration of active virus units in the resuspended precipitate and the original juice from which the precipitate was derived. In two such experiments the ratio of the number of lesions produced by the test (resuspended precipitate) to those produced by the control was 1·06 and 1·228. Although the resuspended precipitate produced more lesions these differences were not statistically significant. (2) Very little active virus remained in the supernatant liquid corresponding to the precipitate, but it was nevertheless measurable, being 5·6 p.c. and 1 p.c. respectively of the control.

The precipitate forms stable solutions when shaken up with buffer solutions with pH values on the acid side and alkaline side of the isoelectric range. Resolution of precipitate and virus go hand in hand, and it is not possible to differentiate between virus and precipitate by selective elution at different pH values. It is therefore clear that the virus is precipitated at pH 3·4, and that it may be resuspended without loss in infectivity.

It is also clear, however, that no very accurate results with respect to percentage precipitation, when this is large, can be obtained by working with the precipitate, since the bulk of experimental work showed that the experimental error is of the order of 10 p.c. to 20 p.c. with the number of replicates commonly used in the present work. In other words, even if we have actually only 85 p.c. precipitation, when the resuspended precipitate is compared with the control the lesion count may not indicate a significant difference, and the estimated precipitation would be 100 p.c. On the other hand, by comparing the supernatant liquid with the control (the experimental error being of the same order) the lesion count would give a value of 15 ± 3 p.c. virus remaining in suspension, and from this a much more accurate estimate of the per cent. precipitation is obtainable (85 ± 3 p.c.). Actually at very low concentration of the virus in the supernatant liquid (high percentage precipitation) the experimental error would exceed the usual 10 to 20 p.c. for optimal concentrations, but in terms of the calculated percentage precipitation it would not vary very greatly. For example, if at 98 p.c. precipitation the experimental error involved in estimating the low concentration of virus in the supernatant liquid is as high as 100 p.c. this would only result in an error of about 2 p.c. in the value calculated. This is in fact about the order of the error near the isoelectric point. Consequently the supernatant liquid has been used throughout to estimate the extent of the precipitation.

Precipitation of the Virus Complex from Clarified Tobacco Plant Juice.

The results obtained by adding clarified juice (obtained from tobacco plants infected with ordinary tobacco mosaic) to buffer solutions at various pH values

are summarized in Tables 1 A, B, and C. The pH values refer to the supernatant liquid after centrifuging. Summaries of the analysis of variance for some of these experiments are set out in Tables 2 A and B.

TABLE 1.

Precipitation of the tobacco mosaic virus complex from buffers at various pH values. The source of virus was clarified juice from artificially infected *N. tabacum* plants. C = lesions from common control inoculum (pH 7). T = lesions from test inocula, prepared by adding portion of supernatant liquid to solutions of pH 7 after treatment at the pH value indicated.

A. Design of experiment. Four 8 × 8 Latin squares on 64 *N. glutinosa* plants trimmed to 4 leaves per plant. The concentration of each inoculum was 1 volume of juice in 1,000 total volume (1/1,000).

pH Value.	3·09		3·20		3·32		3·41		3·53		3·62		3·73		3·84	
	C	T	C	T	C	T	C	T	C	T	C	T	C	T	C	T
No. of lesions on square on																
1st leaves	266	26	166	17	211	15	250	14	259	19	232	20	216	45	177	97
2nd leaves	180	22	339	16	195	9	204	17	255	12	267	22	283	50	263	172
3rd leaves	204	37	173	18	228	23	165	6	163	15	301	24	217	31	212	101
4th leaves	146	19	166	6	147	15	158	11	135	11	115	9	130	25	177	71
$\left(\frac{\text{Test}}{\text{control}}\right) \times 10^2$																
1st leaves	9·78		10·2		7·1		5·6		7·34		8·62		20·8		54·8	
2nd leaves	12·2		4·72		4·62		8·33		4·70		8·24		17·7		65·4	
3rd leaves	18·1		10·4		10·1		3·64		9·20		7·97		14·3		47·7	
4th leaves	13·0		3·61		10·2		6·96		8·15		7·82		19·2		40·1	
Mean ratio × 10^2	13·3		7·2		8·0		6·1		7·3		8·2		18·0		52·0	

B. Design of experiment. Three 6 × 6 Latin squares on 36 *N. glutinosa* plants trimmed to 3 leaves per plant. The concentration of each inoculum was 1/1,500.

pH Value.		2·48		2·80		3·29		3·51		3·86		4·72	
		C	T	C	T	C	T	C	T	C	T	C	T
No. of lesions on square on													
1st leaves		126	135	147	163	132	11	193	28	130	172	205	137
2nd leaves		116	144	147	152	163	12	125	14	129	123	174	110
3rd leaves		111	92	118	138	109	14	87	14	106	111	102	85
$\left(\frac{\text{Test}}{\text{control}}\right) \times 10^2$	1st leaves	107·1		110·9		8·33		14·5		132·3		66·8	
	2nd leaves	124·1		103·4		7·36		11·2		95·3		63·2	
	3rd leaves	82·9		116·9		12·8		16·1		104·7		83·3	
Mean ratio × 10^2		105		110		9·5		13·9		111		71	

C. Design of experiment. Four 4 × 4 Latin squares on 16 *N. glutinosa* plants trimmed to 4 leaves per plant. The concentration of each inoculum was 1/2,000.

pH Value.		2·29		2·92		3·79		4·30	
		C	T	C	T	C	T	C	T
No. of lesions on square on	1st leaves	236	196	209	33	245	33	175	203
	2nd leaves	199	155	231	24	159	39	199	147
	3rd leaves	240	223	88	14	217	19	180	198
	4th leaves	166	159	233	22	94	9	143	151
$\left(\frac{\text{Test}}{\text{control}}\right) \times 10^2$	1st leaves	83·0		15·8		13·5		116·0	
	2nd leaves	77·9		10·4		24·5		73·9	
	3rd leaves	93·0		15·9		8·75		110·0	
	4th leaves	95·8		9·4		9·6		105·6	
Mean ratio × 10^2		87		12·9		14·1		101	

TABLE 2.

Summary of Analysis of Variance of Experiments Recorded in Table 1.

A.

pH Value.	3·09	3·20	3·32	3·41	3·53	3·62	3·73	3·84
Mean difference (C–T)	21·6	24·6	22·5	22·8	23·6	26·25	21·7	12·1

S.E. of each mean = 2·44 For diff. of 6·7 P = ·01 and for diff. of 6·0 P = ·02.
S.E. of diff. between any 2 means = 3·45 For diff. of 9·5 P = ·01 and for diff of 8·5 P = ·02.

* Supplementary tests on individual pairs ('t' tests) 3·09 > 3·20; 3·32 = 3·41; 3·41 = 3·53; 3·53 = 3·62; 3·62 < 3·73; 3·73 < 3·84.

B.

pH Value.	2·48	2·80	3·29	3·51	3·86	4·72
Mean difference (C–T)	−1·0	−2·3	+20·4	+19·4	−2·3	+8·3

S.E. of each mean = 2.31 For diff. of 6·35 P = ·01 and for diff. of 5·7 P = ·02.
S.E. of diff. between any 2 means = 3·26 For diff. of 9·0 P = ·01 and for diff. of 8·0 P = ·02.

Supplementary test on individual pairs ('t' test) 3·29 = 3·51.

* "=" means that the two systems concerned are not significantly different, ">" or "<" means that the two systems concerned are significantly different (P < ·01).

The results of these experiments are represented graphically in Figure 1A. Maximum precipitation is obtained at a pH value near 3·4.

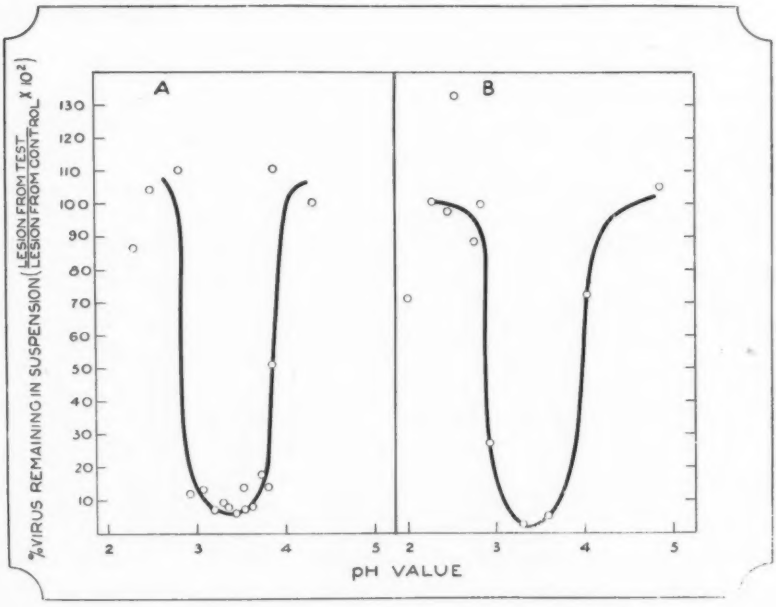

Fig. 1. Precipitation of tobacco mosaic virus at various pH values.
A. From clarified juice of infected tobacco plants.
B. From a suspension of purified virus complex.

Precipitation of Virus Complex from Suspensions of Purified Virus.

Tables 3 A, B, and C summarize the results obtained by using a virus preparation purified by first precipitating it at its isoelectric point and then resuspending this in a buffer solution at pH 7, equal in volume to the clarified juice from which the precipitate was originally obtained. This suspension of purified virus complex was added to buffer solutions of various pH values, and was from then on handled in the same way as described for the clarified juice. A summary of the analysis of variance corresponding with the results summarized in Table 3 is set out in Table 4.

TABLE 3.

Precipitation of the tobacco mosaic virus complex from buffer solutions at various pH values. The source of virus was a suspension of purified virus in a buffer solution at pH 7, the purification being effected by precipitating the virus isoelectrically from clarified juice of artificially infected *N. tabacum* plants. C = lesions from common control inoculum (pH 7). T = lesions from test inocula prepared by adding portions of supernatant liquid to solutions of pH 7 after treatment at the pH value indicated.

A. Design of experiment. Four 8 × 8 Latin squares on 64 *N. glutinosa* plants trimmed to 4 leaves per plant. The concentration of each inoculum was equivalent to 1 volume of juice in 1,000 total volume (1/1,000).

pH Value.	2·50		2·82		3·32		3·62		4·10		5·03		6·05		7·06	
	C	T	C	T	C	T	C	T	C	T	C	T	C	T	C	T
No. of lesions on square on																
1st leaves	190	177	179	164	134	6	123	7	205	53	135	183	180	171	139	142
2nd leaves	197	157	195	208	172	8	220	5	225	39	193	228	175	197	173	192
3rd leaves	155	220	226	230	187	3	218	11	212	51	159	251	175	286	152	212
4th leaves	121	245	122	122	142	1	121	6	162	35	107	197	149	238	109	109
$\left(\frac{\text{Test}}{\text{control}}\right) \times 10^2$																
1st leaves	93·2		91·6		4·48		5·69		25·85		135·6		95·0		102·2	
2nd leaves	79·7		106·7		4·65		2·27		17·3		118·1		112·6		111·0	
3rd leaves	141·9		101·8		1·60		5·05		24·1		157·8		163·4		139·5	
4th leaves	202·5		100·0		0·70		4·96		21·6		184·2		159·8		100·0	
Mean ratio × 10^2	129		100		2·9		4·5		22·2		149		133		113	

B. Design of experiment. Four 8 × 8 Latin squares on 64 *N. glutinosa* plants trimmed to 4 leaves per plant. The concentration of each inoculum was equivalent to 1/1,000.

pH Value.	2·00		2·27		2·57		2·93		3·28		3·95		4·76		5·98	
	C	T	C	T	C	T	C	T	C	T	C	T	C	T	C	T
No. of lesions on square on																
1st leaves	106	74	97	77	89	120	104	26	93	6	49	65	78	128	97	96
2nd leaves	141	80	88	97	92	131	93	28	99	3	160	173	125	139	112	116
3rd leaves	132	100	127	155	103	119	113	18	148	6	158	279	133	166	127	153
4th leaves	132	111	122	112	74	113	100	40	113	3	130	192	128	122	132	143
$\left(\frac{\text{Test}}{\text{control}}\right) \times 10^2$																
1st leaves	69·8		79·4		134·8		25·0		6·45		132·6		164·1		99·0	
2nd leaves	56·7		110·2		142·4		30·1		3·03		108·1		111·2		103·6	
3rd leaves	75·8		122·0		115·5		13·8		4·05		176·6		124·8		120·5	
4th leaves	84·1		91·8		152·7		40·0		2·65		147·7		95·3		108·3	
Mean ratio × 10^2	72		101		136		27·2		4·0		141		124		108	

C. Design of experiment. Three 6 × 6 Latin squares on 36 *N. glutinosa* plants trimmed to 3 leaves per plant. The concentration of each inoculum was equivalent to 1/1,500.

pH Value.		2·45		2·75		3·27		3·57		4·03		4·96	
		C	T	C	T	C	T	C	T	C	T	C	T
No. of lesions on square on	1st leaves	180	172	146	139	169	1	158	15	140	54	193	171
	2nd leaves	130	135	154	122	100	1	96	2	94	59	111	96
	3rd leaves	74	72	95	89	70	0	69	3	116	73	100	105
$\left(\frac{\text{Test}}{\text{control}}\right) \times 10^2$	1st leaves	95·6		95·2		0·59		9·50		38·6		88·6	
	2nd leaves	103·85		79·2		1·00		2·08		62·8		86·5	
	3rd leaves	97·3		93·7		0·0		4·35		62·9		105·0	
Mean ratio × 10^2		99		89		0·46		5·8		54		94	

TABLE 4.

Summary of Analysis of Variance of Experiments Recorded in Table 3.

A.

pH Value.	2·50	2·82	3·32	3·62	4·10	5·03	6·05	7·06
Mean difference (C–T)	−4·25	−0·06	+19·3	+20·4	+19·6	−8·3	−6·7	−2·6

S.E. of each mean = 2·01 For diff. of 5·5 P = ·01 and for diff. of 4·9 P = ·02.
S.E. of diff. between any 2 means = 2·84 For diff. of 7·8 P = ·01 and for diff. of 7·0 P = ·02.

* Supplementary tests on individual pairs ('t' test) 3·32 = 3·62; 3·62 < 4·10; 5·03 = 7·06.

B.

pH Value.	2·00	2·27	2·57	2·93	3·28	3·95	4·76	5·98
Mean difference (C–T)	+4·6	−0·2	−3·9	+9·3	+13·6	−6·6	−2·8	−1·25

S.E. of each mean = 1·49 For diff. of 4·1 P = ·01 and for diff. of 3·7 P = ·02.
S.E. of diff. between any 2 means = 2·11 For diff. of 5·8 P = ·01 and for diff. of 5·2 P = ·02.

Supplementary tests on individual pairs ('t' test) 2·00 = 2·27; 2·93 > 3·28.

C.

pH Value.	2·45	2·75	3·27	3·57	4·03	4·96
Mean Difference (C–T)	0·3	2·5	18·7	16·8	9·1	1·8

S.E. of each mean = 2·04 For diff. of 5·6 P = ·01 and for diff. of 5·0 P = ·02.
S.E. of diff. between any 2 means = 2·89 For diff. of 7·95 P = ·01 and for diff. of 7·1 P = ·02.

Supplementary tests on individual pairs ('t' test) 3·27 = 3·57; 3·57 < 4·03; 4·03 < 4·96.

* "=" means that the two systems concerned are not significantly different, "> or <" means that the two systems concerned are significantly different (P < ·01).

Figure IB shows the result of these experiments graphically. When the pH values of suspensions from different experiments were close to each other these and the value for the amount of virus remaining in suspension have been combined and plotted as one average point. Again the isoelectric point is seen to lie near pH 3·4.

Precipitation of Virus-complex from Clarified Tomato Plant Juice.

Dwarf Champion tomato plants were artificially inoculated with the virus of ordinary tobacco mosaic; the diseased leaves were crushed in a mortar, and the juice expressed and treated as described for tobacco plant juice. The clarified tomato plant juice was used in the same way as the tobacco plant juice for precipitation studies. The results of two experiments carried out at different times with the same stock of juice are set out in Tables 5 A and B, and are shown graphically in Figure 2.

The most acid suspension tested had a pH value of 2·87, so that the curve is not complete on the acid side of the isoelectric point, but since the experiments define this point very well it was not considered worth while carrying the curve further. The analysis of variance is summarized in Tables 6 A and B. The isoelectric point is seen to be identical with that for mosaic virus cultured in tobacco plants.

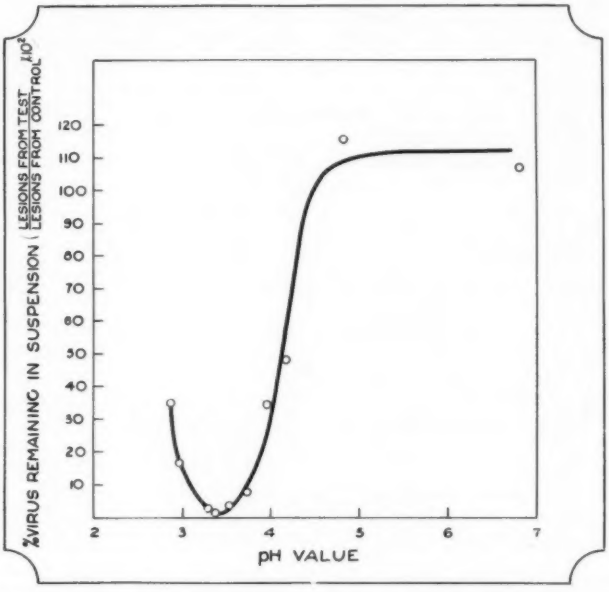

Fig. 2. Precipitation of tobacco mosaic virus at various pH values. The virus was "cultured" in tomato plants, and clarified juice of infected tomato plants was used for the precipitations.

TABLE 5.

Precipitation of the tobacco (or tomato) mosaic virus complex from buffer solutions at various pH values. The source of virus was clarified juice from *L. esculentum* plants artificially infected with the virus of ordinary tobacco mosaic. C = lesions from common control inoculum (pH 7). T = lesions from test inocula prepared by adding portions of the supernatant liquid to solutions of pH 7 after treatment at the pH value indicated.

A. Design of experiment. Three 6 ×6 Latin squares on 36 *N. glutinosa* plants trimmed to 3 leaves per plant. The concentration of each inoculum was 1/1,000.

pH Value.		2·96		3·28		3·52		3·74		3·95		4·17	
		C	T	C	T	C	T	C	T	C	T	C	T
No. of lesions on square on	1st leaves	210	37	264	10	239	2	171	14	250	90	261	160
	2nd leaves	223	33	197	3	163	6	233	13	272	62	238	102
	3rd leaves	174	29	173	6	170	11	228	16	190	74	316	135
$\left(\frac{\text{Test}}{\text{control}}\right) \times 10^2$	1st leaves	17·6		3·8		0·8		8·2		36·0		61·3	
	2nd leaves	14·8		1·5		3·7		5·6		22·8		42·9	
	3rd leaves	16·7		3·5		7·1		7·0		38·9		42·7	
Mean ratio × 10^2		16·4		2·9		3·9		6·9		33		49·0	

B. Design of experiment. Three 4 × 4 Latin squares of 16 *N. glutinosa* plants trimmed to 3 leaves per plant. The concentration of each inoculum was 1/1,000.

pH Value.		2·87		3·37		4·82		6·78	
		C	T	C	T	C	T	C	T
No. of lesions on square on	1st leaves	281	91	229	1	189	226	145	192
	2nd leaves	96	41	108	3	96	114	213	184
	3rd leaves	107	32	97	2	108	118	144	147
$\left(\frac{\text{Test}}{\text{control}}\right) \times 10^2$	1st leaves	32·4		0·44		119·6		132·4	
	2nd leaves	42·7		2·78		118·7		86·4	
	3rd leaves	29·9		2·06		109·3		102·1	
Mean ratio × 10^2		35		1·8		116		107	

TABLE 6.

Summary of Analysis of Variance of Experiments Recorded in Table 5.

A.

pH Value.	2·96	3·28	3·52	3·74	3·95	4·17
Mean difference (C–T)	28·2	34·2	30·7	32·7	27·0	23·2

S.E. of each mean = 3·55 For diff. of 9·75 P = ·01 and for diff. of 8·7 P = ·02.
S.E. of diff. between any 2 means = 5·02 For diff. of 13·8 P = ·01 and for diff. of 12·5 P = ·02.

* Supplementary tests on individual pairs ('t' test) 2·96 > 3·28; 3·28 = 3·52; 3·52 < = †3·74; 3·74 < 3·95; 3·95 < 4·17. † P approx. ·02.

B.

pH Value.	2·87	3·37	4·82	6·78
Mean difference (C–T)	26·7	35·7	—5·4	—1·75

S.E. of each mean = 5·74 For diff. of 15·8 P = ·01 and for diff. of 14·1 P = ·02.
S.E. of diff. between any 2 means = 8·11 For diff. of 22·3 P = ·01 and for diff. of 19·9 P = ·02.

Supplementary tests on individual pairs ('t' test) 2·87 > 3·37.

* "=" means that the two systems concerned are not significantly different, ">" or "<" means that the two systems concerned are significantly different (P < ·01).

Proportion of the Juice Represented by the Precipitate and Some Preliminary Chemical Tests.

The precipitate consists of about 0·3 p.c. by weight of the crude juice. In three experiments carried out at different times with juice from different sets of plants, the isoelectric precipitate (dried in a vacuum desiccator over $CaCl_2$) was found to be 0·30, 0·24, and 0·38 p.c. of the crude juice, and for the first two this corresponds to 2·92 p.c. and 3·74 p.c. of the total solids respectively (the total solids were not determined for the third).

The precipitate containing the virus gives a positive reaction for the following tests: biuret, xanthoproteic, Millon-Cole, Adamkiewicz, Rosenheim's and Cole's aldehyde tests. The precipitate therefore contains protein, and the protein contains tyrosine and trytophane. Solubility relationships of the precipitate class it as a globulin. The nitrogen content of the desiccator dried precipitate without further purification was found to be 14 p.c. (Kjeldahl). The problem is complicated by the fact that a precipitate is obtained at the same pH value from the juice

of healthy plants. However this precipitate exhibits important differences from that derived from the juice of virus-containing plants. The material is at present being subjected to a detailed chemical investigation, and in collaboration with Mr. G. C. McLennan, veterinary pathologist of the Stock and Brands Department, to serological tests. The results of this work will be reported later.

Note on Tests for Significance.

Reference to the mean differences and to differences between means and their respective standard errors (Tables 2, 4, and 6) will give a clear indication of significant differences over the transition zones on either side of the isoelectric point, and need not be further discussed. The orthodox method of analysis of variance for the Latin square which has been used to construct these tables is, however, not valid in estimating significant differences near the isoelectric point because the standard errors of the low counts are smaller than the one calculated for the whole experiment, and the differences (C–T) are derived from control counts which are uniformly high, and 'test' counts which vary from high to low numbers according as we are far from or near to the isoelectric point. The net result is that in the low count region differences between any two means often do not appear to be significant, whereas when analyzed on the 'test' counts alone in individual pairs (the corresponding control values of which are not significantly different) by Fisher's 't' test, they are shown to be clearly significant. The advantages of the tables as a means of reducing the amount of data and subsequent discussion, and their essential correctness, makes them indispensable. However, in order to make good this deficiency in the main portions of the tables, a further section has been added at the end of each table giving results of tests on individual pairs of test values by the 't' test. The author is at present co-operating with Mr. E. A. Cornish of this Institute, who is planning a modification of the usual system of analysis of variance in order to correct the deficiency mentioned above, thus making the estimation of error more exact, and at the same time eliminating the necessity for the above supplementary tests.

DISCUSSION.

It is clear that the virus-complex is precipitated to various extents over the pH range 3 to 4, and that the point of maximum precipitation is near pH 3·4. The three curves are similar in essentials, and the point of maximum precipitation is seen to be the same in all cases. Precipitation carried out on numerous occasions and at widely separated times have always given the same results. The isoelectric point may, therefore, be regarded as constant at pH $3{\cdot}4 \pm 0{\cdot}1$.

There are some differences, however, which are of interest. For example, near a pH value of 4 the results are rather more erratic than at other pH values, particularly is this so for the purified virus. This may in a large measure be accounted for by the fact that this is the steepest part of the curve and borders the transition zone. Nevertheless a virus precipitate which has been resuspended in a buffer solution is never transparent (like the clarified juice from which it was precipitated) at the concentration of the stock suspension (equivalent to the concentration in the original juice). It is only when higher dilutions are used that these suspensions are transparent. The inocula, for example, prepared from the precipitate are quite clear and colourless. The juice is therefore a better stabilizer than we have been able to supply. The suspensions of purified virus in buffer solution at

the optimum pH value of 7 appear, therefore, to be inherently less stable than the suspensions of the virus in the tobacco juice, and this may account in some measure for the wider spread of the curves on the alkaline side of the isoelectric point. This wider spread is also noticeable for the "tomato" mosaic curve, and in this connection it is noteworthy that suspensions of virus in clarified tomato juice are not as stable as similar suspensions in tobacco juice. The former become cloudy (under storage at 1° C.) very much sooner and to a more marked degree than the latter.

Reference to the tables shows that in several instances the number of lesions resulting from inocula prepared from suspensions which have been treated with buffer solutions just outside the pH range where precipitation takes place, is significantly larger than the number resulting from the control inocula. This is by no means a constant phenomenon, and the increases are not very large, compared with the probable error of estimation. They may therefore be due to an abnormally high experimental error in these cases. On the other hand, the phenomenon has occurred rather too frequently to dismiss it without further thought, and it may be due to a greater dispersion of virus aggregates or to an aggregation of small sub-critical aggregates to larger ones, by this means increasing the number possessing the critical value for lesion production, while not decreasing this number to the same extent by the further aggregation of previously existing critical aggregates. The whole question is a complex one, and is receiving attention. In the meantime the main thesis is not affected.

The isoelectric point is the same whether the starting material is the unpurified plant juice or a suspension of the purified virus-complex, and whether the virus is cultured in tobacco or tomato plants. Although these two types of plants belong to the same family, they are very different in habit and in many other respects. From the circumstance that the isoelectric point is independent of the host plant we may conclude that the property is a characteristic of the virus itself or of a complex (e.g. a virus-protein complex) in which the virus and some other plant constituent (the latter being common to the various hosts) are fundamentally related. The latter view would afford a simple explanation of the specificity of the virus, which would on this view only attack those plants which possessed the "common plant constituent" referred to above. The possibility that the virus may be carried down by some unrelated constituent of the juice cannot be entirely ruled out, but the above facts, taken in conjunction with the facts that the precipitation is practically quantitative and that the virus cannot be brought back into suspension either on the acid or on the alkaline side of pH 3·4 without the precipitate also, lead to the conclusion that the precipitate is wholly virus or something of the nature of a virus-substrate complex.

It is possible that the virus itself is something in the nature of an altered protein, in which another reactive group (not normal to the protein) has been produced through some agency at present unknown, and that such a protein can produce a similar change in other proteins having a similar structure: and so the

process of proliferation goes on. On account of this new reactive group the altered protein (the virus or virus-complex) enters into reactions which are not normal to the metabolic processes, and by thus upsetting the normal metabolic processes brings about the "disease", with its attendant symptoms. It is possible, of course, that this reactive protein may be a *normal* constituent of some other plant (or animal), and only causes trouble when introduced into plants to which it is not a normal constituent but which contain a closely allied protein (or other substance), which then forms the substrate for proliferation and causes metabolic upsets.

SUMMARY.

1. The virus of ordinary tobacco mosaic is precipitated from the clarified juice of infected tobacco plants to various extents over the pH range 3 to 4. The point of maximum precipitation is at pH 3·4, where, under suitable conditions, more than 99 p.c. of the virus may be precipitated.

2. The precipitate, which contains practically the whole of the virus, constitutes about 0·3 p.c. of the clarified juice or 3 p.c. of the total solids of the crude juice. On an average the yield is 2 milligrams per millilitre of juice.

3. Relatively stable colloidal solutions of the precipitate are obtained by eluting with buffer solutions of pH values between 2·8 and 2·3 or between 4·5 and 7·5. The resultant solutions contain the whole of the precipitated virus. It is not possible by selective elution to separate the virus from the precipitate, either on the acid or on the alkaline side of its isoelectric point. From the evidence as a whole it is concluded that the precipitate is the virus itself or a complex of virus with some fundamentally related substance present in the juice.

4. Precipitation of the virus from suspensions of the "isoelectric" precipitate in buffer solution of pH 7 takes place in the same way as from the juice, maximum precipitation being at pH 3·4.

5. The juice of tomato plants artificially infected with the same virus behaves similarly and maximum precipitation occurs at the same pH value. The isoelectric point of the virus-complex may therefore be taken as pH $3{\cdot}4 \pm 0{\cdot}1$.

6. The precipitate gave positive tests for protein, and desiccator dried samples contained 14 p.c. nitrogen.

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THE EFFECT OF METABOLIC INHIBITORS ON THE THERAPEUTIC IRRADIATION OF MOUSE TUMOURS[1]

by

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(Submitted for publication 26th November, 1935.)

One of us (W.M.) has developed a technique for the investigation of methods by which a tumour might be sensitized *in vivo* to the action of X-radiation. Briefly, a series of tumour-bearing mice are given a constant X-ray dose, and some animals receive preliminary treatment with the object of securing more favourable effects. It has been shown that a preliminary heating of the tumour region by means of a diathermy current may result in a more pronounced X-ray effect. Preliminary tuberculin injection also appears to have a similar favourable effect, whilst potassium iodide proved to be inactive. This work is being published separately.

In the search for substances which might be more active we considered the work of Crabtree and Cramer (1933), who found that the radio-sensitivity of tumour tissue *in vitro* may be modified by substances which affect cellular respiration. Working with tumour slices they found that immersion in physiological saline solution, of M/500 and M/1,000 concentration in potassium cyanide, reduced the respiration by 85 to 95 p.c. Treatment with this concentration of cyanide caused no permanent injury to the tissue, the effect being completely reversible when the tissue was restored to a normal physiological solution. On exposing tumour slices in such cyanide solutions to radium radiation the sensitivity of the tissue was found to be much increased as compared with control slices in solutions free from cyanide. On the other hand anaerobiosis which diminishes respiration in a different manner was found to decrease radio-sensitivity.

The possibility of increasing the radio-sensitivity of cells by introducing suitable chemical substances is in agreement with results obtained in our laboratory on chemical reactions induced by X and gamma radiations. It has been shown that some of these reactions can be greatly influenced by the addition of quite small amounts of suitable chemical reagents (Harker 1934, a, b). The effect of X and gamma radiations on living tissues must be regarded as due mainly to chemical changes produced by the radiations.

[1] This work was carried out under the control of the Cancer Research Committee of the University of Sydney, and with the aid of the Cancer Research and Treatment Fund.

Our first object was to test whether cyanide could be introduced into the tumour of the living animal in order to determine whether its radio-sensitivity would be increased as found by Crabtree and Cramer *in vitro*.

Lethal Dose of Cyanide.

Throughout this investigation white laboratory mice were used, and tumours were furnished by implanting the sarcoma S-37 of the Imperial Cancer Research Fund. Taking a mouse 25 grammes in weight and assuming even distribution of cyanide, it would be necessary to inject 1·6 mg. of potassium cyanide (0·75 c.c. of M/30 cyanide in isotonic saline used throughout by us) in order to obtain a concentration of M/1,000 as employed by Crabtree and Cramer. It was found impossible to give this dose owing to the well-known toxic action of cyanide on the nervous system. By injection into a tail vein it was found that the lethal dose was considerably less than 0·1 c.c. of our M/30 solution. Accordingly methods of administration were tried which might delay absorption.

Intraperitoneal and subcutaneous injections of the above order were also rapidly fatal. Finally we tried a modified subcutaneous injection into the distal part of the tail, avoiding the veins. This delayed absorption sufficiently to permit doses of 0·2-0·4 c.c. being tolerated. These amounts caused extreme disturbance of respiration for a few minutes, but the animal appeared normal after half an hour.

Preliminary Analytical Work.

It was obviously of prime importance to determine exactly how much cyanide was present in tumours excised at varying times after a sublethal dose. It is well known that a tumour has a poor blood supply, and from this point of view it might be expected that very little cyanide would be absorbed by it. On the other hand it has been stated by Cushing that tumour tissue is more permeable to salts than normal tissue.

It was first necessary to find a method for the estimation of minute quantities of prussic acid in tissues. For this purpose the method of Chelle (1919) seemed highly suitable. In this the tissue is macerated with water, rendered acid with phosphoric acid, and distilled. The prussic acid in the distillate is concentrated by removing it with a sufficiently large current of air, and absorbing it in a small volume of decinormal caustic potash solution. In this latter solution it is estimated by the ammoniacal silver iodide reagent. The merit of the method lies in the concentration process, which is very effective. Tests were made by adding small known quantities of cyanide (0·01 mg. to 0·1 mg.) to tumour tissue which had been ground with sand and water. This treatment separated the cells without disintegrating them. The results obtained were distinctly low, owing to destruction of nearly half the prussic acid in the preliminary distillation process—a destruction which takes place also, but to a lesser extent, in the absence of tissue. The percentage loss though was found to be fairly constant, and an allowance could be made for it, leaving but a small margin of error. This was the method adopted for the estimation of prussic acid in the tumour and other tissues examined. A short account of the investigation by one of us leading to the adoption of this method has already been given (Harker, 1935).

Estimation of Cyanide in the Tissues of the Living Animal.

In applying the above method 0·2 c.c. of the M/30 cyanide solution (0·43 mg. KCN) was injected into the tail tissues of tumour-bearing mice as described, a procedure which does not

involve any risk of direct contamination of regions later subjected to analysis. After varying intervals the animal was killed with ether, and the tumour was shelled out for analysis. The liver and muscle were also analysed for control purposes. Muscle was taken as a typical tissue with a good blood supply, while the liver is mainly supplied by the complex portal system.

In the following table the results are stated as percentage absorptions. From the weights of tumours, liver, and muscle, the proportionate amounts of the total cyanide injected were calculated. If all the cyanide were distributed evenly throughout the animal and there were no loss of cyanide in the tissue, these amounts would represent 100 p.c. in each case. The amounts of cyanide found in the tissues analysed are given then as percentages of these injected amounts.

TABLE 1.

Absorption of Cyanide by the Tumour, Liver, and Muscle of Mice injected with 0·2 c.c. of an M/30 Solution of Potassium Cyanide.

Time.	Weight of Tumour.	Per cent. Absorption.	Average.†	Weight of Liver.	Per cent. Absorption.	Average.	Weight of Muscle.	Per cent. Absorption.	Average.
10 mins.	1·37	4·4	5·3	1·56	6·3	5·7	1·08	9·1	7·4
,,	1·10	6·1		1·97	5·0		1·30	5·7	
,,	2·17	3·9							
1 hour	0·89	16·7							
,,	1·18	11·5		1·69	10·4				
,,	1·03	11·2	11·4	1·40	9·6	10·3	1·14	11·8	8·6
,,				0·84	11·0		1·5	5·4	
,,	2·08	7·8							
,,	*2·46	4·7							
,,	*3·58	1·9							
3 hours	1·63	8·3	6·6	1·37	7·6	6·2	1·02	7·3	6·0
,,	1·54	4·8		1·55	4·8		1·05	4·6	
24 hours	1·80	trace		1·78	0·0		1·53	0·0	
,,	2·14	0·0		2·25	0·0		1·55	0·0	

* These tumours contained a considerable necrotic centre. The analysis refers to the healthy periphery. The central portion on analysis showed no cyanide.

† The averages in this column are given for medium weight tumours only.

Variation of Absorbed Cyanide with Tumour Size.

It was soon apparent in the case of the tumours that the percentage absorption, i.e. the absorption per unit weight, depended greatly on their size. This is clearly shown by the figures in Table 1 for tumours of different sizes, where the absorption is highest in small tumours, probably on account of their better blood supply. In the case of the two largest tumours examined massive necrosis had taken place in the central portions, and these portions were separated, the figures stated referring to the outside healthy parts of these tumours. The central portions on analysis showed no cyanide. A diagrammatic representation of the effect of size of tumour on absorption is given by the vertical line on the right of the graph in Fig. 1. Here small tumours mean those under 1 gramme in weight, medium 1-2 grammes, large over 2 grammes, and very large over 3 grammes. In comparing the absorption of tumour tissue with liver and muscle it was decided

to employ tumours of 1-2 grammes in weight owing to the above-mentioned variations.

Variation of Absorbed Cyanide with Time after Injection.

Table 1 shows the figures obtained for absorption for different intervals of time after injection. It will be seen that the results vary in the same manner for tumour, liver, and muscle. There was an increase in absorption between ten minutes and one hour, but the amount of cyanide present was distinctly less in

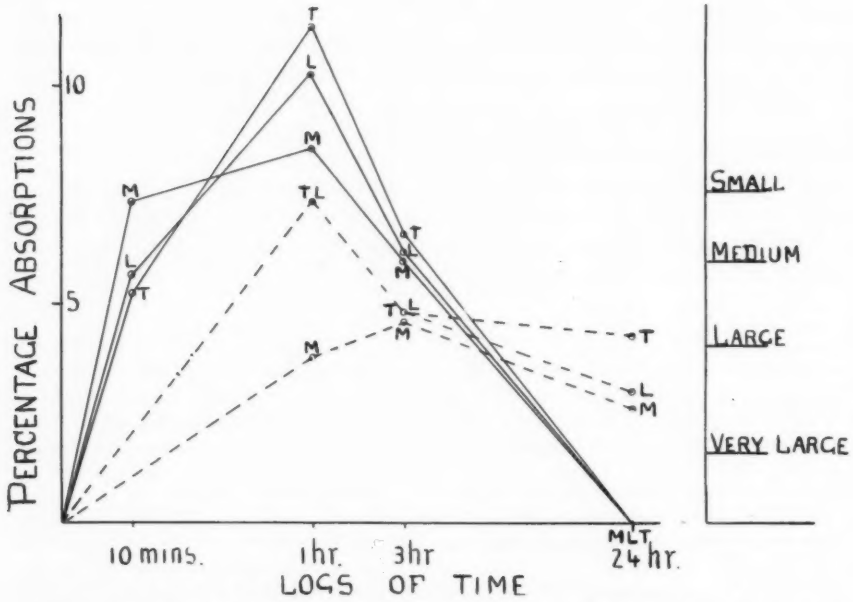

Figure 1. The percentage absorptions, when potassium cyanide was injected, are shown by the continuous lines in the graph, whilst the broken lines indicate the absorption with the double cyanide of silver and potassium. T = tumour, L = liver, M = muscle. On the right, drawn to half scale, are shown the relative absorptions by different sized tumours when potassium cyanide was injected.

three hours than in one hour, and had disappeared altogether after 24 hours. The average figures for these different periods of time are shown by the continuous line in Fig. 1 which depicts the absorption of cyanide by the three types of tissue. It will be noted that there was not a great deal of difference in the amount absorbed by the three different kinds of tissue. At the one hour period the tumour gave the highest maximum.

Fate of Injected Cyanide.

Since the quantity of cyanide found in the tissues examined was never more than 16·7 per cent. of the amount injected, assuming even distribution (Table 1), the question arises as to the fate of the cyanide in the body. The point was investigated first by mincing up a whole mouse and adding a known quantity (0·038 mgm.) of potassium cyanide. This was about one-tenth the quantity injected into the living mouse. Water was added and the mouse tissue ground up with sand and analysed as before. Only 5·7 per cent. of the added cyanide was recovered. In other words 94·3 per cent. had been destroyed. The blank experiments on tumour tissue already mentioned had indicated only a comparatively slight loss due to the addition of this tissue; and this was re-checked under the present conditions (see Table 2). To trace the matter further individual organs were removed from the animal and treated with the same quantity of cyanide as was added to the whole animal. The results are given in Table 2, and show that the brain, weight for weight, caused the disappearance of over 60 times as much cyanide as tumour tissue.

TABLE 2.

Destruction of Cyanide by Different Tissues during the Process of Analysis.

Potassium Cyanide (0·038 mgm.) was added in each case before analysis.

Tissue Examined.	Weight of Tissue in grammes.	Per cent. added Cyanide. Found.	Destroyed.
Whole Mouse	20	5·7	94·3
Tumour	4·0	90·0	10·0
Stomach and Intestines	3·36	44·4	55·6
Brain	0·39	36·6	63·4
Liver and Muscle	4·44	58·9	41·1

This throws an interesting light on the well-known toxic effect of cyanide on the nervous tissue of the animal. In the process of analysis the mixture of cyanide and tissue is acidified and heated to distil off the prussic acid. It is not suggested that this treatment is the same as bringing the cyanide into contact with the tissues at blood temperature in the living animal, but it does indicate that certain tissues, e.g. brain, are much more potent than others in combining with the cyanide to the extent that prussic acid is no longer recoverable from it on acidification. In the case of the mixed liver and muscle, the muscle tissue was doubtless responsible for the major portion of the destructive effect, as evidence had been obtained that liver tissue had not much effect in this direction.

Degree of Inhibition of Respiration.

Since 0·75 c.c. of M/30 cyanide solution, when evenly distributed, would give an M/1,000 concentration in cyanide in a full-grown mouse, the concentration actually obtained in the tumour after one hour was $\frac{M/}{1000} \times \frac{11{\cdot}4 \times 0{\cdot}2}{100 \times 0{\cdot}75} = \frac{M}{1000} \times \frac{3{\cdot}04}{100}$ that is only 3 per cent. of the concentration employed by Crabtree and Cramer in

their *in vitro* experiments. In the above calculation no notice has been taken of the amount of solid matter present. This means that the concentration would be slightly higher than 3 per cent.

Crabtree and Cramer found an inhibition of 85-95 p.c. of the respiration in employing solutions of M/1,000 and M/500 potassium cyanide. We have no data as to the inhibitory effect of a concentration of cyanide 3/100 × M/1,000. There is no reason to suppose that the respiration would be reduced in proportion to the lowering of the concentration, because Crabtree and Cramer found that a concentration of M/20 could be employed without permanently damaging the cells and the inhibition could not have been noticeably greater than with M/1,000. The positive results we obtained with radiation point to a definite reduction in respiration, although the concentration of cyanide was so low.

Use of the Double Cyanide of Silver and Potassium.

It has been shown (Table 1, Fig. 1) that the amount of cyanide in the tumour falls away fairly rapidly after the first hour. Our attention was directed to the possibility of using some combination of cyanide which would retain the cyanide ions for a longer period in the tumour tissue. It was hoped in this way to obtain a higher concentration of cyanide in the tumour during irradiation and for some time afterwards. The double cyanide of silver and potassium, $KAg(CN)_2$, was prepared and made up to M/30 solution. The solution was injected in the manner previously described, and the three tissues, tumour, muscle, and liver were analysed. The results are given in Table 3.

TABLE 3.

Absorption of Cyanide by the Tumour, Liver, and Muscle of Mice injected with 0·2 c.c. of an M/30 Solution of Potassium Silver Cyanide.

Time.	Weight of Tumour.	Per cent. Absorption.	Average.	Weight of Liver.	Per cent. Absorption.	Average.	Weight of Muscle.	Per cent. Absorption.	Average.
1 hour	1·78	7·4		1·79	7·4		1·70	3·8	
3 hours	1·54	4·8		1·55	4·8		1·05	4·6	
24 hours	1·17	4·9	4·3	1·33	3·2	3·0	0·83	2·0	2·6
	1·82	3·7		2·07	2·7		1·48	3·2	

The method of analysis only detects the cyanide present as KCN in the KCN, AgCN complex, so that if the KCN, as was most probably the case, carried along with it its quota of AgCN, there was present in the tissue just double the quantity of CN indicated by analysis. The important point brought out by the analytical results is that the double salt persists in the tissues for a much longer period than the simple salt, and is found in quite considerable quantity at the end of 24 hours. This is depicted by the broken lines in Fig. 1. An anomaly in these results is shown in the figures obtained for 1 hour and 3 hours' absorption in the case of muscle, the

latter being higher than the former. The figures obtained for the muscle tissue all through were more irregular than those for the two other tissues, possibly because muscle tissue is subject to considerable and rapid alterations in blood supply.

Preliminary Experiments with Glycolytic Inhibitors.

The work of Lundsgaard, Euler, and others has shown that iodo-acetic acid, whilst having little effect on the oxidative metabolism of carbohydrates, exercises a powerful inhibition on the fermentative or glycolytic process. Thus Lundsgaard (1930) showed that alcoholic fermentation by living yeast and by zymase was completely inhibited by employing sodium-iodo-acetate in concentration of 1/5,000, whereas the oxygen consumption was little affected. Experiments were extended to frogs in which 3·4 mg. of iodo-acetate per gram body weight were injected into the dorsal lymph sac, and it was shown that after a short period no lactic acid was found in the muscles, whereas oxygen consumption was not much affected. Lundsgaard pointed out that as the metabolism of cancer tissue differs from normal, in that there is always considerable glycolysis, it should be possible to change the metabolism of tumour tissue to approximate that of normal tissue by applying sodium iodo-acetate. He, therefore, made daily injections of iodo-acetate into tumour-bearing mice, but, owing to the poisonous character of the substance and its highly irritating effect, he was unable to give any conclusions regarding it, except to say that the experiments were worth continuing. The fact that in cancer tissue several molecules of carbohydrate are converted to lactic acid for every one oxidized stimulates investigation with an agent which can suppress the glycolytic function.

Some time ago we attempted a modification of Lundsgaard's work by administering sodium iodo-acetate in the drinking water over a period of about fourteen days. However, growth experiments failed to show any difference compared with controls. In a later series of tests with irradiated animals no modification in sensitivity was observed. The tumours of the treated animals were then analysed for iodine by Kendall's (1914) method with negative results. We wish to express our indebtedness to Mr. W. Bishop, M.Sc., former Bosch Cancer Research Fellow, for his kindness in carrying out the major portion of this analytical work. It is probable that the iodo-acetic acid is destroyed in the stomach before absorption can take place.

Crabtree and Cramer (1932) later published their experiments already referred to, in which they showed that the radio-sensitivity of tumours was not modified *in vitro* by inhibiting glycolysis with sodium iodo-acetate and sodium fluoride. They used M/10,000 sodium iodo-acetate, which they showed practically inhibited glycolysis without causing any lasting detrimental effect. Nevertheless we determined to carry out irradiation experiments on the living animal after injecting the iodo-acetic acid intravenously, since the conditions were different to those obtained *in vitro*. For this purpose tumour-bearing animals were given intravenously 0·4 c.c. of an M/160 solution of sodium iodo-acetate. This was the amount calculated to give a concentration of M/10,000 in the animal assuming even distribution.

Irradiation Experiments after Injection with Cyanide and Iodo-acetic Acid.

We now proceeded to investigate whether either cyanide or iodo-acetic acid in the concentrations mentioned would exert any effect on the X-ray sensitivity of the tumour *in vivo*.

The technique was based on that of work (W.M.) already mentioned which is being published elsewhere. Briefly the tumours were implanted into a series of mice from a small homogeneous piece of parent tumour. About seven days later the diameters of the new tumours were estimated, and three were selected of uniform small size, or if this was impossible, a variation not exceeding 1 millimetre was allowed. These initial sizes are recorded in columns 2, 3, and 4, Table IV.

TABLE 4.

Radiation of Tumours of Mice injected with solutions of Potassium Cyanide and Sodium Iodo-Acetate.

Experimental Number.	Initial Size of Tumour: mm.			Ratio Final Tumour Diam. / Initial Tumour Diam.			Final Weight of Tumour.			Body Weight of Mouse.		
	C	CN	1A	C	CN	1A	C	CN	1A	C	CN	1A
1	5	5	5	4	4	6	1·89	1·51	3·97	20	15	20
2	2	3	3	9	5	5	1·11	1·33	1·70	14·5	22	16
3	8	8	7	3	2	3	3·1	1·32	1·55	19·5	24	24
4	5	5	5	3	2·5	1·5	1·34	1·22	0·46	26	24	21
5	2	2	2	4	5	5	0·28	0·71	1·05	19·5	23	24·5
6	5	4	5	2	2·5	1	0·83	0·48	0·30	16	17	17
Average	4·5	4·5	4·5	4·2	3·5	3·6	1·43	1·10	1·50	19	21	20
Control Experiments Mice Injected but not Radiated.												
1	3	3	2	5	5	6·5	1·99	1·52	0·96	14	22·5	16·5
2	3	4	4	3	7	7	0·62	2·86	4·02	21	13·5	12·5
3	5	4	4	3·5	4	4·5	2·68	1·55	1·62	15	21·5	13·5
4	7	8	8	3·5	2·5	2·5	2·56	2·49	2·18	21	14·5	19·5
Average	4·5	4·7	4·5	3·75	4·6	5	1·95	2·1	2·2	18	18	15·5

One of the selected mice was marked Control (C) and another, marked Cyanide (CN), received 0·2 c.c. of our stock cyanide solution subcutaneously in the tissues of the tail as already described. This was given half an hour before irradiation, to permit of a full recovery of respiration. A normal respiration was desirable since Crabtree and Cramer have shown that anaerobiosis acts in the opposite way to that of cyanides. It should be noted that partial asphyxia in the animal is really a combination of anaerobiosis and increased CO_2 tension. The third animal selected received 0·4 c.c. of the stock iodo-acetic solution in a tail vein (IA). This was irradiated without delay in conjunction with the control and cyanide experiments. The three animals were stitched to a frame which was placed in a nearly uniform part of the X-ray field, and the tumour region was exposed for 1 hour through ports 1 sq. cm. in area. The potential was 120 kV., the milliamperage 10, the focal distance 63 cm., and 1 mm. aluminium filter was employed, whilst a Gaiffe-Gallot intensiometer indicated a dose of 530 r. This has been found (W.M.) to retard tumour growth by about 33 p.c., an optimum condition for observing any additional effect following cyanide or iodo-acetic acid injection.

Control experiments (see Table 4) were made to determine the action of cyanide and iodo-acetic acid without X-ray treatment. As before, three mice were stitched to a frame to reproduce the factor of trauma in the irradiation experiments. A somewhat greater initial variation in the size of the tumours was allowed,

but it will be seen that the average initial size in each of columns 2, 3, and 4 (Table 4) was practically constant.

The animals were examined frequently from the time of implantation to check any abnormal tumour growth, but it has only been necessary to record the final diameter, or rather the ratio of the final to the initial diameter 10 days after irradiation (see columns 5, 6, and 7, Table 4).

With careful implantation this tumour grows in a nearly spherical form for a time, and the method of taking diameters may thus replace the more tedious chartings which cannot be easily reduced to numerical values. The tumour may also be shelled out from its bed after reflecting the skin, and this prompted us to attempt making a record of the final weight (Table 4, columns 8, 9, and 10). The gravimetric method accounts for all three dimensions of the tumour, and we consider it to be the more reliable. Unfortunately it is not possible to determine an initial weight, a quantity which one ignores on the understanding that the initial diameter measurements are small and reach an equal average for each column. On inspecting the tables it will be seen that the gravimetric results in a large number of instances correspond with the first power of the growth ratios, and not with their cubes as might be expected. Thus both methods give results of the same order. We are unable to suggest the reason for this anomaly, but it is probably connected with the flattening from central necrosis and fibrous shrinkage observed in many of the tumours. Table 4 (columns 11, 12, and 13) also gives approximate carcase weights after the tumours had been removed.

TABLE 5.

Radiation of Tumours of Mice injected with Solutions of Potassium Silver Cyanide and Sodium Iodo-Acetate.

Experimental Number.	Initial Size of Tumour: mm.			Ratio Final Tumour Diam. / Initial Tumour Diam.			Final Weight of Tumour.			Body Weight of Mouse.		
	C	CN	1A	C	CN	1A	C	CN	1A	C	CN	1A
1	4	3	3	3	4	4	0·74	0·55	0·60	21·5	21	20
2	4	3	5	4	4	3	1·12	1·01	0·66	12	13	11·5
3	3	5	3	8	2	4	1·74	0·32	0·67	13·5	12·5	15
4	5	4	4	3	3	2·5	1·27	1·45	1·09	18	12	15
5	3	3	4	7	4	4·5	1·36	0·65	0·98	11·5	16·5	12
Average	3·8	3·6	3·8	5	3·4	3·6	1·24	0·80	0·80	13·3	15	14·7
Average for Tables IV & V	4·15	4·05	4·15	4·6	3·45	3·6	1·35	0·95	1·15	16	18	17
	Control Experiments Mice Injected but not Radiated.											
1	5	5	3	4	3	5	2	0·94	1·43	8·5	10·5	12
2	4	4	5	6	6	5	3·2	3·31	3·52	10·5	10	9
3	4	4	2	5	6	10	1·51	1·86	1·32	17	18	18
4	5	5	8	4	4	3	2·34	2·25	3·97	19·5	13	13·5
Average	4·5	4·5	4·5	4·75	4·75	5·75	2·46	2·09	2·56	13·9	12·9	13·1
Average for Tables IV & V	4·5	4·6	4·5	4·25	4·7	5·4	2·2	2·1	2·4	16	15·5	14·3

The data in Table 5 were obtained in a similar manner except that the cyanide solution was replaced by the silver cyanide salt already mentioned, and an interval of 4 hours was allowed after injection to permit of complete respiratory recovery. Analyses quoted above show that the tumour retained almost the maximal amount of cyanide at this period. Owing to the prolonged toxic action of this salt it was found advisable to reduce the amount injected to 0·15 c.c. of stock solution for mice of normal weight, whilst in some very light animals (see Table 5) the amount injected was further reduced in proportion to the body weight. Control results for non-irradiated animals are also given.

DISCUSSION OF RESULTS.

On inspecting the results it will be seen that there are more serious irregularities in Table 4 than the later results in Table 5. This irregularity is attributed to a shortage of mice necessitating breaks in the work and the use of different breeds, whilst the results in Table 5 were obtained rapidly with mice from one source. The results may be considered together, and it will be seen that radiation alone reduced the average weight at the end of the observation period from 2·2 gm. to approximately 1·35 gm.

It will be seen from Table 4 that the average weights of the irradiated tumours were 1·1 gm. with cyanide and 1·43 gm. without this treatment. Thus the simple salt KCN increased the X-ray shrinkage by 23·1 p.c. In the case of the double salt KCN, AgCN (Table 5) the weights were 0·80 gm. and 1·24 gm. respectively, a difference of 36·6 p.c., indicating a more powerful action. As previously mentioned, the results obtained by the ratio method were of the same order.

Contrary to the results of Crabtree and Cramer the iodo-acetic acid also appears to increase X-ray action. With the ratio method this appears in both tables, whilst the gravimetric method only shows the effect in Table 5. The latter result is the more reliable owing to the irregular growth of the tumours in Table 4.

It was essential that the cyanide or iodo-acetic acid should have no effect in the absence of radiation. Otherwise one would be merely investigating the combined result of two injurious stimuli, and it would be impossible to associate any beneficial action with respiratory or glycolytic interference. On inspecting the control figures in the lower portions of Tables 4 and 5 it will be seen that the non-irradiated cyanide and iodo-acetic acid experiments show approximately the same growth as the controls, although obviously the order of accuracy would not permit of the detection of small differences.

The broad objective of this work is to find a substance which may provide a considerable sensitizing effect when a tumour receives therapeutic irradiation. Results are valuable whether positive or negative, but when positive it is desirable to know whether substance A is more effective than B. Iodo-acetic acid and cyanide in the concentrations permissible in the living animal appeared to have a

distinct action, but this action falls short of an ideal activator for X-ray irradiation. The effect is of the same order as that obtained by one of us (W.M.) in the case of tuberculin injection and diathermy treatment.

In the case of cyanide Crabtree and Cramer found a pronounced radiation effect with a M/1,000 concentration. Since our experiments show a definite increase in radio-sensitivity with 3 per cent. of this concentration, it appears that cyanide might be a powerful activator to X-radiation in the living animal if it were possible by protecting the nervous system to employ higher concentrations.

CONCLUSIONS.

1. The injection of cyanides into the living animal has been found to increase the radio-sensitivity of tumour tissue in confirmation of the *in vitro* experiments of Crabtree and Cramer.

2. Inhibition of glycolysis following the injection of iodo-acetic acid into the living animal also appeared to cause an increased radio-sensitivity. Crabtree and Cramer found no such effect *in vitro*.

3. Oral administration of iodo-acetic acid gave negative results, but in this case analysis failed to give any evidence of absorption.

4. The amount of cyanide absorbed by the tumour, liver, and muscle tissues of mice injected with solutions of potassium cyanide and the double potassium silver cyanide has been determined.

5. The double salt persisted in the tissues for a much longer period than the simple salt.

6. It has been shown *in vitro* that unit weight of brain tissue destroys much more cyanide than other tissues examined.

7. It is indicated that radio-sensitivity could be considerably increased if larger concentrations of cyanide could be obtained in the tumour of the living animal by protecting the nervous system from the highly toxic action of this compound.

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INDUCED LYSOGENICITY AND MUTATION OF BACTERIOPHAGE WITHIN LYSOGENIC BACTERIA[1]

by

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In the course of work on staphylococcal bacteriophages, we have encountered a curious relation between two phages, one of which was apparently a mutant derivative of the other. The only essential difference seemed to be that the original phage produced resistant cultures with extraordinary facility while the presumed mutant behaved in the normal fashion of any moderately active bacteriophage. When we attempted to elucidate the nature of this difference, attention was first concentrated on finding correlated differences between the two phages. No such differences were found. The nature of the resistant cultures then became the centre of interest. It was found that all the resistant forms appearing after the action of the original phage were lysogenic, producing at first phage of the "original" type. As the culture aged, the "mutant" phage made its appearance along with the "original"

Such relationships between phages, in the clear-cut form of this example, are unique in our experience. Similar, if less striking phenomena, however, have been recorded for other phages and bacteria, and the data obtained in the present work may perhaps be of value to some of those interested in the interactions between phage and susceptible bacterium.

The phages in question, C and C′, were described by Burnet and McKie in 1930, but the nature of their difference was not then investigated. Phage C was obtained from rodent faeces and lysed only a white coccus SF, isolated from the same source. The phage produced very little clearing in broth culture, though filtrates gave fairly good plaque count titres. The plaques were highly characteristic in appearance, very large for a coccal phage, about 3-4 mm. in diameter and of annular form, the centre being invariably occupied by a heavy growth of bacteria, and the clear ring only a little more than a millimetre across. An unusual but constant feature of the plaques is that they extend their diameter when the plate is incubated for periods beyond 24 hours. To some extent the central bacterial growth also expands, but this lags relatively to the lysis, and the sum effect

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is chiefly a widening of the ring of lysis. Phage C′ made its appearance after the phage had been under study for some months, in the form of certain plaques identical with the normal ones except for the absence of the central growth of bacteria (Fig. 1). The plaques when isolated bred true, and phage C′ has maintained this characteristic since.

At the time of its appearance, no large plaque staphylococcal phage other than C had been isolated in the laboratory, so that the possibility of contamination can be ruled out. The two phages are serologically identical (Burnet and Lush 1935) and the further relations to be discussed below will make it clear that there is a true genetic relation between them: C′ must be regarded as a mutant of C.

The Type of Resistant Cultures Produced by the Two Phages.

There is no difficulty in obtaining resistant cultures after the action of these two phages. If two plates are spread with a young culture of SF, and when this has dried a few drops of C and C′ spread on respective plates, after incubation the first will show a thin line of lysis marking the edge of the area on which the phage was spread (see Fig. 2), but over the rest of the area there is a confluent sheet of apparently normal culture. This central growth, however, is completely resistant to phage C and retains its character on subculture: it will be referred to as SF/C. The plate spread with C′ shows the usual appearance obtained after lysis with a moderately strong phage. There is an area of complete clearing on which are scattered a few well-developed resistant colonies and an occasional semilysed "nibbled" colony (Fig. 3). The intact colonies breed true and retain their resistance: they will be referred to as SF/C′.

The properties of SF, SF/C, and SF/C′ may now be compared briefly. All show a similar type of growth on broth or agar, and the colonies are indistinguishable from one another by inspection. The effect of various phages lysing SF on the two variants is shown in Table 1, which shows both the lytic effect on agar and the absorptive power of killed bacteria for the phages.

TABLE 1.

Characteristics of Initial and Phage-resistant Cultures.

	Lytic action of phages shown						Absorption of phages C and C′ by dead cultures. Phage C.			Phage C′		
Culture.	A	B	C	C′	D	Au I	2	20	200	2	20	200
SF	+	+	+	+	+	+	100%	60%	0	80%	40%	0
SF/C	+	+	−	−	+	+	0	0	0	0	0	0
SF/C′	+	−	−	−	+	+	0	0	0	0	0	0

+ = active lysis on agar, − = no lysis.

Absorption of phages by bacteria was tested according to the technique previously described (Burnet and Lush 1935), serial dilutions of heat-killed broth cultures being added to small constant amounts of phage, and the mixtures plated

after 4 hours at 37° C. The percentage reduction in plaque count is shown in the table.

The essential point to be noted is that SF/C is also resistant to the action of phage C′. SF/C′ is resistant to both C and C′ but differs from SF/C in being also resistant to phage B. These reactions are paralleled by the phage absorption results obtained with dead bacteria. Serologically no difference could be detected between SF and SF/C by cross absorption tests, but SF/C′ is serologically distinguishable from SF by the same technique (Burnet and McKie 1930).

Most of the experiments to be described depend on the fact that organisms of SF/C type are never *produced* by phage C′, but when present can multiply normally in the presence of excess of this phage. This makes it possible to study fairly closely the influence of phage C in converting SF organisms into the heritable modification SF/C.

The Production of SF/C by Phage C.

It is obvious from the appearance of its plaques that phage C provokes resistance with the greatest of ease. This can be established quantitatively in a number of ways. If one plates a series of dilutions of SF culture on normal agar plates and on plates spread with concentrated phage C, the number of colonies is identical within the limits of experimental error in the two series, but on the phage spread plates the colonies are all of SF/C type.

In another type of experiment we have plated serial dilutions of C with SF as in a plaque titration, but, instead of allowing plaques to form, the areas are respread with strong phage C′ within half an hour of mixing C and SF. After incubation we find that the number of resistant colonies on each area is roughly proportional to the concentration of phage C, and in some experiments approximates to the number of plaques obtained in a normal phage titration. A typical protocol is as follows:

Experiment 1. Plates of nutrient agar were spread with 0·1 c.c. of a 4 hour broth culture of SF. When these had dried, 0·02 c.c. quantities of serial dilutions of phage C in broth were spread on sectors according to the usual technique in phage titrations. Fifteen minutes later each sector was spread with a drop of undiluted phage C′ to cover the whole of the area on which the C phage dilution had been placed. The plates were again allowed to dry, and incubated over night.

TABLE 2.

Relation between Concentration of Phage C and the Numbers of Resistant Colonies induced.

Concentration of phage C.	Colonies appearing in the presence of excess of phage C′
10^{-2}	Semi-confluent growth.
10^{-3}	500 ±
10^{-4}	120, 151
10^{-5}	26, 28
Nil	18, 12

TABLE 2A.

Type of Resistant Colonies Produced.

Sources of colonies (see Table II).	Number of colonies.	Lytic action of phages. B	C	C′	D	Type of resistant culture.
Phage C 10^{-5}, excess C′	11	+	—	—	+	SF/C
Phage C 10^{-5}, excess C′	9	—	—	—	+	SF/C′
Excess phage C′ alone	10	—	—	—	+	SF/C′

Table 2 shows the number of colonies which developed on each sector. In the absence of phage C about 15 colonies resistant to C′ appeared, and we can assume that on each of the other sectors about that number are also due directly to the influence of C′, the remainder representing the number of organisms which were rendered resistant to phage C before they came into effective contact with phage C′. This assumption could be tested directly as far as it applied to the sectors spread with 10^{-5} dilution of C. Table 2A shows that of 20 colonies tested from these sectors, approximately half were of the SF/C type, the others being SF/C′. In the absence of phage C all the colonies were as was to be expected of the SF/C′ type. At a dilution of 10^{-5} then, phage C produces under these conditions 13 or 14 resistant bacteria. The same quantity of phage would give rise to 20-50 plaques as judged by previous titrations, but this was not actually determined in this experiment.

Several other experiments were carried out to determine the ratio between the number of plaques produced under normal conditions and the number of resistant colonies obtained in the present type of experiment. Table 3 shows the results of four experiments using the same batch of phage C, and essentially the same technique as in Experiment 1. Certain variations were made in the time and conditions of contact between phage C and the sensitive culture before C′ was added, but they seemed to have no significant effect, and will not be discussed. In general the results show that allowing for the Dreyer and Campbell Renton effect the number of resistant colonies is roughly proportional to the amount of phage present, and, that as a rule, about 10-20 p.c. of phage particles capable of giving rise to a plaque will, under these conditions, induce the appearance of a resistant colony.

TABLE 3.

Relation between Plaque Count and Number of Resistant Colonies.

Phage C concentration.	Plaque count.	Average number of colonies in each experiment. Phage C′ being added 15 minutes after phage C. 1	2	3	4
10^{-3}	—	158	320	185	±450
10^{-4}	±500	60	38	18	54
10^{-5}	78	14	5·5	2·0	11
2×10^{-6}	16	—	3·0	2·6	6·0
Nil	—	4	1·25	0·5	3·25

There is no necessity that phage C′ should be added subsequent to the action of C. Figures 2-4 show plates spread with a 1 : 100 dilution of a broth culture of SF and, subsequently, spread over a central area with (Fig. 2) phage C (Fig. 3) phage C′ and (Fig. 4) a mixture of the two. It will be seen that there are about 100 times as many colonies arising on (4) as on (3). Even if phage C is added 15 minutes after C′ there is a large increase in the number of resistant colonies over those produced by C′ alone.

In considering the time relationship in these experiments, it must be remembered that neither phage is very actively adsorbed to the susceptible organism, either when killed by heat (see Table 1) or in the living state. Even in the presence, as in all these experiments, of excess of bacteria it may be an hour or longer before any given phage particle makes effective contact with a susceptible organism. During this period the bacterial population is also of course increasing in density, so that the conditions are too complex for any strictly quantitative interpretation of the results.

Certain points are, however, clearly established; the most important is that the appearance of SF/C resistant colonies is due to the direct positive effect of phage C on the coccus SF and not to a selection of pre-existent variants. This latter alternative is in most examples of resistant production by phage very difficult to exclude, and is often apparently the most important factor (Burnet 1929). A glance at Figs. 3 and 4 show at once that, while the colonies on 4 (mixed phages) are obviously resistant to C′, 99 p.c. of the organisms from which they arose were not initially resistant to C′, since they fail to produce colonies on the plate spread with this phage only.

The change from SF to SF/C under the influence of phage C must in some cases at least occur quite rapidly, probably within an hour, if the descendants are to be protected against the action of undiluted C′ phage. Further, the experiments indicate that a remarkably high proportion of effective contacts between phage and bacteria must result in the induction of resistance rather than lysis. The difficulties associated with the slow adsorption of phage to bacteria made it impossible to give any numerical estimate of this proportion, but it seems very unlikely that it is less than 5 p.c. On the evidence that when SF is plated on agar covered with strong phage C just as many resistant colonies appear as susceptible ones on ordinary agar, we were at first inclined to consider the possibility that 100 p.c. of contacts resulted in the appearance of resistant forms, phage multiplication taking place by some other process than the usual one. Probably a majority of the authors who have studied the question consider that in the initial stages of the action of phage on a susceptible culture, multiplication of the lytic agent proceeds in the absence of any bacterial lysis. Bronfenbrenner (1928) in fact claims that there is a distinct growth-stimulating effect in the early stages. One of us (Burnet 1934) has argued that there is no experimental basis for this point of view, and

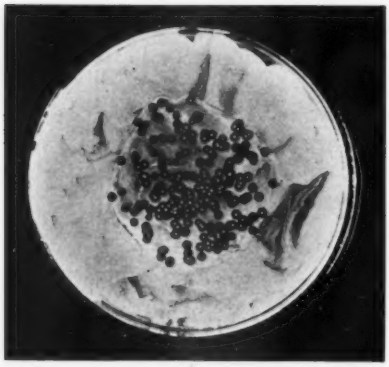

Fig. 1.

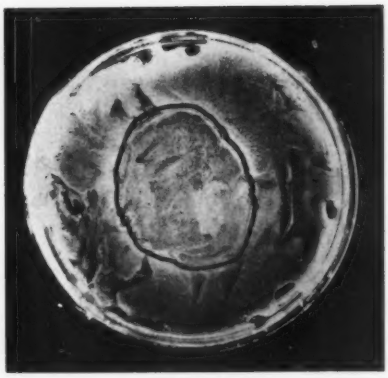

Fig. 2.

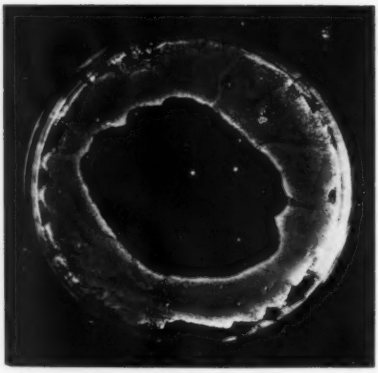

Fig. 3.

Fig. 4.

that all the published experimental data are compatible with the hypothesis that increase in amount of phage results only from the disruption of infected bacteria. *A priori* it seemed likely that if there were any exceptions to this rule the action of phage C on SF would be one of them. Phage C′ on the other hand was obviously a typical bacteriophage—producing only about one resistant colony per million bacteria lysed—and was known from previous experiments to multiply in normal fashion. A series of experiments was therefore carried out on the functional activities of phage C, mostly in comparison with C′, in order to discover whether it showed any fundamental peculiarities of behaviour.

In their general intrinsic qualities the two phages are practically identical; serologically they are indistinguishable. Both are relatively resistant to the photodynamic action of methylene blue and to inactivation by strong urea solution, and both are unaffected by citrate in the medium. Functionally, too, there is extremely little difference between the two phages, apart from the greater readiness of C to produce resistant variants. On agar, plaques of each type can just be distinguished about 2 hours after spreading. The two types develop at the same rate, and the central bacterial growth of C is only clearly visible after about 5 hours. When lysis by the two phages is studied by direct microscopic observation on the agar surface, in each case first lysis is observed about 1¼-1½ hours after mixing. With C, many more bacteria give rise to microcolonies than with C′, but typical disappearance of the bacterium by lysis is readily seen, and appears to be quite similar to that induced by C′.

Multiplication of phage C in broth follows the ordinary course, with, however, a longer initial lag than is found with dysentery phages.

Experiment 2. A tube containing a growing broth culture of SF was seeded with sufficient phage to give 2 or 3 plaques when 0·02 c.c. was plated. It was maintained at 37°C. and at suitable intervals eight such volumes were plated on agar. The plaque counts were as tabulated (Table IV), each figure being the average of eight counts.

TABLE 4.

Multiplication of Phage C in Broth at 37° C.

	Immediately.	30 min.	45 min.	60 min.	75 min.	90 min.
Average plaque count	2·0	1·75	2·4	2·25	23·0	160

Using the method of single particle multiplication (Burnet 1930) the same explosive increase occurred as is characteristic of all phages which have been

EXPLANATION OF TEXT FIGURES.

Fig. 1. Plate inoculated with SF and spread with a dilute mixture of phages C and C′. The plaques produced by phage C show central resistant growth while those of phage C′ have a clear centre.

Figs. 2, 3, and 4. Plates spread with 0·1 c.c. of 24 hour broth culture of SF diluted 1 : 100. After drying, 0·02 c.c. of the phages shown was spread over the central area. Fig. 2. Phage C undiluted stock filtrate. Fig 3. Phage C′ undiluted stock filtrate. Fig. 4. A mixture of equal parts of phages C and C′.

studied by this technique. The best of three consistent experiments gave the plaque counts shown in Table 4A with phage C. In all experiments the first increase indicated that 80-150 phage particles were liberated from each infected bacterium, lysis occurring between 70 and 90 minutes after mixing. A suitable modification of this experiment showed that a similar explosive multiplication took place on the surface of agar, although in this instance the first increase appeared later, between 90 and 105 minutes.

TABLE 4A.

To Show Explosive Multiplication of Phage C.

Time of plating after phage and culture mixed.	Plaque counts obtained from each tube.							
60–65 minutes	0	1	0	0	0	3	0	0
65–70 ,,	0	1	1	0	0	2	1	0
70–75 ,,	2	0	0	1	0	0	1	1
75–80 ,,	1	1	0	0	1	0	1	2
80–85 ,,	1	0	1	1	–	–	–	–
85–90 ,,	1	0	1	154	1	0	1	136
90–95 ,,	0	0	186	0	0	79	0	2

Lysogenesis of SF/C.

The resistant culture SF/C which is so readily induced by the action of phage C is consistently lysogenic, producing in young culture phage of C type, but in older cultures it *gives rise to plaques of both C and C′ types.* Both types like the original ones breed true after isolation and passage on SF.

A series of cultures of a strain of SF/C which had been re-isolated three or four times since its induction by phage C, was set up in broth (pH 8·0) and kept at 37° C. Each day the lysogenicity was tested by filtering portion of a culture through a gradocol membrane (A.P.D. 0·8μ) and plating both filtrate and unfiltered culture on plates spread with SF. Details of one such experiment are given in Table 5.

TABLE 5.

Lysogenesis of SF/C Cultures of Different Ages.

Time.	Unfiltered SF/C.	Gradocol membrane filtrate SF/C.
1 day	1×10^6, all C type	—
2 days	1×10^8, all C type	3×10^6, all C type
4 days	2×10^5, 1% C′ type	2×10^5, 2% C′ type
5 days	5×10^7, 0·5% C′ type	—
6 days	2×10^8, 50% C′ type	2×10^8, 50% C′ type
7 days	5×10^7, 1% C′ type	—

In the majority of experiments C′ appeared somewhat earlier, usually on the third day, a maximum mutation being reached on the fourth or fifth day. The test shown in Table 5 was carried over a longer period of time, and a marked drop in the amount of C′ present was noted on the seventh day.

Apart from the change in type of the liberated phage with ageing of the culture, the strain SF/C behaved as regards lysogenesis in the same fashion as any classical lysogenic strain, e.g. the enteritidis strain BTM described by Burnet and McKie (1929). Lysis of the strain by an unrelated phage failed to liberate any detectably greater amount of phage. In these experiments phage Au1 was used (Burnet and Lush 1935), and after lysis titration of the C or C′ phage present was carried out in the presence of excess of antiphage serum specific for phage Au1. The strain also resembled BTM in that when it was rendered resistant to another phage (in this case phage B) it still retained its characteristic lysogenicity, nor could the lysogenic quality be removed by successive subculture of SF/C in the presence of an antiphage serum highly active against C and C′.

Some differences were observed in the time taken for phage C′ to appear from different SF/C cultures, and with one the change in type failed to occur. A number of experiments were made in an attempt to control the rate of appearance of the variant phage, but no significant results were obtained.

The transformation of the incorporated C phage into C′ which occurs as the culture ages is a phenomenon of great interest, but very difficult to interpret. A clue is perhaps provided by the fact that in the later platings one frequently encounters plaques which show both C and C′ characteristics. In every case such double plaques indicate that from what was originally a C plaque a C′ variant has appeared and given rise to a sector of C′ clearing in the plaque. Change from C′ to C does not occur. It is reasonable to assume that the C → C′ change in this instance is via the resistant SF/C form which is developing in the centre of the growing plaque. The phage particle which initiates the appearance of such a composite plaque is clearly different from those present in stock phage C filtrates or liberated from young cultures of SF/C in virtue of this greater ease with which the C → C′ transformation occurs. In some sense at least it must be regarded as an intermediate unstable form.

There are indications from two different directions that the liberation of phage C′ from ageing cultures of SF/C is always associated with the destruction of the coccus from which it is derived. Unless this were the case, platings from unfiltered cultures on the susceptible strain SF would almost certainly show some C′ plaques in which there was a distinct single colony of SF/C. If we consider a single coccus lodging on the plate and within its first 3 or 4 generations giving rise to phage C′ the appearance resulting would be a typical C′ plaque with a single central colony, not the irregular accumulation of secondary colonies visible with a lens in the central region of a C plaque. Such plaques were carefully looked for but never seen. It is probable therefore that when the phage within a given coccus has developed the potentiality of giving rise to a C′ plaque, it destroys the coccus by lysis as soon as the latter is placed in or on fresh media in which growth can occur.

In the second place if we could convert the lysogenic strain SF/C by some

means not involving the use of phage C′ into a variant showing the same type of resistance as is shown by the natural non-lysogenic resistant strain SF/C′, one might expect to annul the production of phage C′ if its liberation were dependent on actual lysis of the bacterium by internally appearing phage C′. Reference to Table 1 will show that by the action of phage B on SF/C a variant having SF/C′ characters can be obtained. Two such variants SF/C/B were prepared and broth cultures tested over a period of 8 days. In neither instance did any C′ type plaques appear. On one occasion a single plaque of a new type was found. This showed a clear centre, then a very narrow ring of secondary growth, then the usual clear outer zone. Phage derived from this plaque bred true to type, consistently giving plaques of similar character. In general this strain C″ more closely resembled C than C′, giving resistant variants of the SF/C type which were lysogenic, producing phage of C″ type. The point to be stressed is that in the doubly resistant strain produced by the action of phage B on SF/C, no mutation of phage C to C′ was observed. It seems as if the resistance induced by B on SF/C (or the almost similar resistance of the non-lysogenic SF/C′ obtained by the direct action of C′ on the original SF culture) is of a more fundamental character than the resistance to C′ shown by SF/C. The most reasonable interpretation is that SF/C resists C′ because of a surface change preventing effective attachment of the phage, but is incapable of preventing its lytic action should it develop within the bacterium from the incorporated C phage. With the more resistant variants SF/C/B there is an intrinsic resistance to C′ irrespective of whether attack is from without or within or alternatively the change C to C′ is completely inhibited.

DISCUSSION.

This investigation arose out of a desire to elucidate the striking difference in appearance between the plaques of phage C and its mutant C′, and has led us considerably further than we had anticipated. The actual form taken by the phenomena is almost unique as far as can be judged from the literature and our own experience, and any deductions are not immediately applicable to any other types of bacteriophage activity. In the course of the investigation, however, a number of points were established which may be of sufficient general interest to workers with bacteriophages to warrant publication.

In the first place the extraordinary ease with which phage C produced resistant variants suggested strongly that if any phages were capable of multiplication without lysis of the sensitive bacterium this should be one of them. The experiment illustrated by Table 4A shows conclusively that like every other phage that has been tested, phage C multiplies explosively, 80 or more fresh phage particles being liberated from a disrupted bacterium from 60 to 90 minutes after phage was added to the culture. This provides a fresh confirmation of the correctness of d'Herelle's original view of the nature of phage multiplication.

Secondly, the production of the resistant lysogenic strain SF/C provides a clear-cut example of the direct positive induction of change in bacterial character by the action of a bacteriophage. In this instance the alternative of selection by phage from pre-existent variants in the population submitted to lysis is definitely excluded. The rapidity with which the change is induced is noteworthy. Within an hour of contact with phage C the surface of the bacterium has changed so that it no longer adsorbs either phage C or C′ and becomes insusceptible to their action. This changed character is then transmitted indefinitely to its descendants. It is not possible to say whether this surface change results from an altered genetic constitution of the bacterium or is directly induced by the associated phage at each generation. According to Wollmann's hypothesis the distinction between the two alternatives would disappear, the phage being regarded as a gene re-introduced into the genetic make-up of the organism.

Finally, we have the demonstration of a definite mutation of a bacteriophage occurring in the course of lysogenesis. Most accounts of bacteriophage mutations are open to the same objections as can be raised against the claims to have produced phage from normal bacterial cultures. In the present instance the mutation appears to affect only one aspect of the phage—that which determines the relative proportion of effective contacts which give rise to lysis and resistance respectively —the other recognizable characteristics remaining unchanged. Change in the character of a phage incorporated in a lysogenic strain has recently been described by d'Herelle and Rakieten (1934) and, although there is rarely any evidence of increase in vigour in these cases, the C to C′ change cannot be regarded as unique.

SUMMARY.

A bacteriophage (C) acting on the non-pathogenic white coccus SF is characterized by the unusual frequency with which it provokes the appearance of resistant forms. Each plaque shows a central growth of resistant culture and suitable experiments indicate that under the usual conditions of growth 10 or 20 p.c. of effective contacts between phage and susceptible bacterium result in the appearance of resistant lysogenic variants, the remainder initiating lysis in classical fashion. The resistant culture shows no gross evidence of lytic action on agar, but in broth culture liberates considerable amounts of phage. In young cultures this phage is of the original type, but as the culture ages a variant phage C′ makes its appearance. This produces plaques of ordinary appearance without the central resistant growth, and gives only a small number of predominantly non-lysogenic resistant colonies after lysis of agar cultures. In all other respects, serological character, for example, the variant phage is indistinguishable from the parent phage C. Certain aspects of these phenomena are briefly discussed.

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FURTHER OBSERVATIONS ON THE CHEMISTRY OF *d*-NOR-NICOTINE. AN ALKALOID OF *DUBOISIA HOPWOODII*

by

C. STANTON HICKS

(From the Department of Human Physiology and Pharmacology, University of Adelaide).

(Submitted for publication 24th January, 1936.)

The original researches leading to the identification and isolation of d-nor-nicotine from the leaves of *Duboisia Hopwoodii* have been reported in this journal by Hicks and LeMessurier (1935); whilst Späth, Hicks, and Zajic (1935) have firmly established the chemical constitution of the alkaloid.

The present communication extends the work of these workers.

Preparation of the Iodo-methylate.

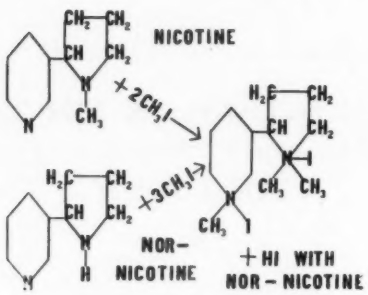
0·5 gm. of the base were dissolved in 2 c.c. methyl alcohol and 2·5 gm. methyl iodide were added, followed by a solution of 0·2 gm. pure potassium hydroxide in 2·5 c.c. methyl alcohol. Crystals of potassium iodide separated, and after 48 hours the supernatant liquid was decanted. On adding one gram of methyl iodide, a solid crystalline cake rapidly formed on stirring. The mass was rubbed up with ether to remove traces of an oily residue, and dried in air to give a yield of 1·3 gm. Melting point, 208–209°C.

Analysis:

5·49 mg. : 2·2 mg. H_2O 6·51 $MgCO_2$
5·00 mg. : 0·250 CCN. N.T.P.
4·52 mg. : 2·575 MgI.
Methylimid estimation (Pregl-Edelbacher)
5·212 mg. : 8·73 mg. AgI. (10·5% CH_3)
Calculated % $CH_3 = 10·11$
Calculated composition for C_{12}, H_{20}, N_2, I_2

	C 32·29	H 4·52	N 6·28	I 56·91
Found	C 32·00	H 4·40	N 6·92	I 57·00

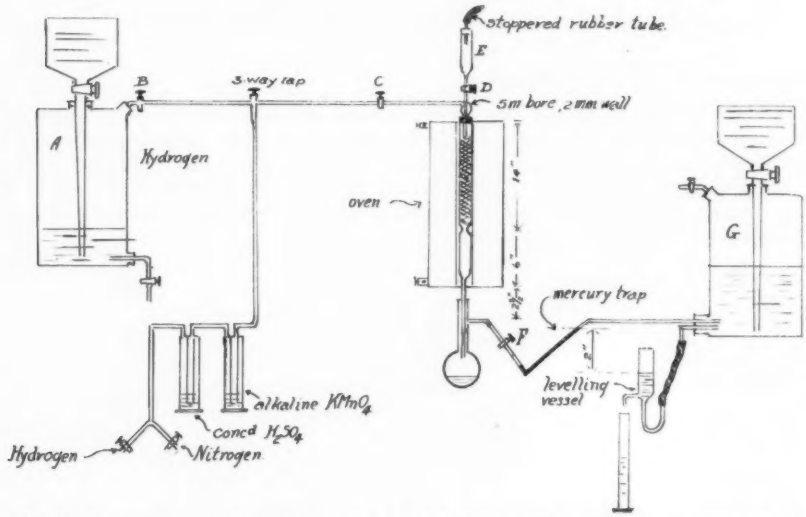
The iodomethylate of nicotine was prepared according to Ehrenstein (1931), using a specially pure specimen provided by Professor Ernst Späth; and the mixed melting points showed no depression. The following scheme shows the constitutional changes involved. M.P. 208·5-209·5°C.



Catalytic de-hydrogenation of d-nor-nicotine.

Using the method of Wibaut (1928) slightly modified from Ehrenstein (1931).

The reaction tube had a wall 2 mm. thick, a bore of 1·5 cm., and was some 58 cm. in over-all length. It was constricted at 36 cm., and tapered off at 49 cm.—this being the length of the portion in the electric resistance oven. The upper portion (see diagram) contained the platinized asbestos, which was made according to Loew-Zelinsky (1924), and found by Ehrenstein to work satisfactorily. After the apparatus had been filled with hydrogen and the temperature of the oven raised to 320–330°C., the alkaloid was forced slowly in from E by squeezing on the rubber tube, q.v. This operation required about two hours, during which the hydrogen displaced was



Modified arrangement of apparatus for the dehydrogenation according to Wibaut, after Ehrenstein.

The catalyst is 33% platinum asbestos prepared according to Loew-Zelinsky. Dehydrogenation takes place in an atmosphere of hydrogen, and the last traces of residuum are swept into the collecting flask by a stream of that gas, during cooling from 330°C. The hydrogen is finally replaced by nitrogen.

collected over water in G by displacement, the volume being measured in terms of water displaced. This amounted to 278 c.c. of hydrogen at N.T.P. Tap C was then closed, and the apparatus allowed to cool in a current of hydrogen maintained for 3 hours to drive most of the remaining fluid down the reaction tube. The hydrogen was finally replaced by nitrogen. The 1·05 gm. of viscous yellow fluid was again passed through the reduction chamber, to ensure complete dehydrogenation, and during this stage a further 60 c.c. of hydrogen was liberated, making a total of 338 c.c. measured at N.T.P. Calculated hydrogen = 402 c.c.

The reaction tube was finally washed out with ether, filtered, and added to the distillate and dried over anhydrous Na_2SO_4. The ether was evaporated in a stream of nitrogen, leaving a yield of 0·805 gm. Following Ehrenstein's procedure for the "nicotein" fractions (1931), the residue was distilled in a Claisen flask with two capillaries, one below and the other above the surface of the liquid, but we took the precaution of distilling in an atmosphere of nitrogen. A constant boiling point of 194·2°C. was obtained at a pressure of 30 mm. Hg., with bath temperature of 235°C. The small amount of resinous residue was rejected. The yellow viscous oil was run, by warming, into a crystallizing dish which was placed in a desiccator filled with nitrogen, where it rapidly crystallized. M.P. 94°–96·2°C. Readily soluble in ether, insoluble in petroleum ether.

The substance was re-crystallized from a mixture of ether and petroleum ether to give large, needle-shaped crystals. M.P. 100–102°C.

After drying over P_2O_5 at 66°, the following results were obtained:

Analysis:

5·05 mg. : 13·3 mg. CO_2 2·65 mg. H_2O

4·001 mg. : 0·619 c.c. Nitrogen at N.T.P.

Calculated Percentage Composition for C_9, H_8, N_2:

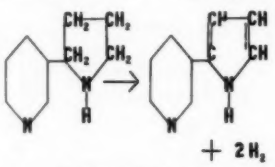
C 74·96, H 5·60, N 19·44

Found C 71·40, H 5·82, N 19·37

The picrate was prepared by adding saturated picric acid in alcohol, to a dilute alcoholic solution of the substance. It crystallized after agitation in square plaques. M.P. 202–203°.

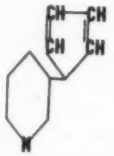
DISCUSSION.

It was shown by Ehrenstein (1931a) that the pyrollidine ring can practically be dehydrated to pyrrol—*cf.* Wibaut (1930), and also Zelinsky (1931). It had previously been shown by Wibaut (1928) that the N-methyl group was not detached by such methods—*i.e.* that the ring did not open. Similarly, it has been shown by Ehrenstein (1931) that 2 methyl-4 phenyl-pyrollidine could be so reduced to the corresponding pyrrol. This proves that the CH_3 group shows no tendency to enter and widen the ring. He found that the lower boiling fraction of the two obtained from nicotine, on being passed over platinum asbestos at 320-330° C., gave in accordance with the above 2-[β-pyridyl]-pyrrol, thus:



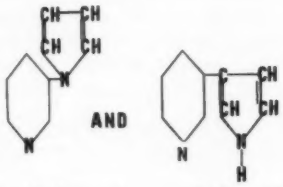
2-[β-pyridyl]-pyrrol

This substance readily crystallizes and gives a M.P. 100-102°. Picrate crystals M.P. 202-203° C. Pictet (1895) reports a similar compound in the course of his classical researches into the alkaloids of Nicotiana, but named it N-[β-pyridyl]-pyrrol.



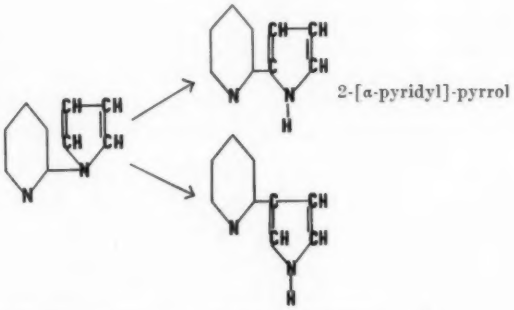
N-[β-pyridyl]-pyrrol

He again reported it later (1904) as appearing during a step in the synthesis of nicotine, and by passing N-[β-pyridyl]-pyrrol through a red-hot tube. According to the method used, Pictet should have obtained a substance meeting at 72°, yielding a picrate M.P. 182. The difference between Pictet's compound and that obtained by Ehrenstein remains unexplained, unless it was a mixture of:



N-[β-pyridyl]-pyrrol 3-[β-pyridyl]-pyrrol

Wibaut (1923) has recently described the conversion of N-[α-pyridyl]-pyrrol by Pietet's method into such a mixture of isomers, thus:



N-[α-pyridyl]-pyrrol 3-[α-pyridyl]-pyrrol

From the results of our dehydrogenation, therefore, we may conclude that we have obtained N-[β-pyridyl]-pyrrol, thus confirming the evidence in favour of a nor-nicotine constitution for the Duboisia alkaloid, and supporting Ehrenstein's work on the lower boiling fraction from "Nicotein".

It was shown by Späth, Hicks, and Zajic (1935) that the Duboisia alkaloid as extracted by Hicks and LeMessurier (q.v.) was a mixture of some 62 p.c. dextro and racemic compounds. This may be due to the extensive decomposition which part of the leaves underwent, as a result of wet warm weather experienced during the expedition to Central Australia, upon which the material was collected. Partial resolution of a racemate may thus be the explanation, and the matter is being further investigated at present. A complete study of the pharmacology of the alkaloid has been reported elsewhere.[1]

SUMMARY.

The iodomethylate of the chief alkaloid from *Duboisia Hopwoodii* is identical with that obtained from pure l-nicotine.

The methylimid estimation according to Pregl-Edlbacher, confirms this.

Dehydrogenation of the alkaloid over platinized asbestos at 330° gave rise to N-[β-pyridyl]-pyrrol.

These results confirm the previous work, pointing to the existence of nor-nicotine as the chief alkaloid of *Duboisia Hopwoodii*.

ACKNOWLEDGMENT.

My thanks are due to Dr. T. A. Henry, Chemical Director of the Wellcome Research Foundation for his valuable advice in connection with these investigations.

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AN EXTRACT FROM THE ADRENAL GLAND CAUSING LUTEINISATION OF THE OVARIES AND ENDOMETRIAL HYPERPLASIA

by

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(Submitted for publication 2nd January, 1936.)

PREPARATION AND CHEMISTRY OF THE EXTRACT (Russell Allen).

The whole adrenal glands were removed from the animal as soon after death as possible, sliced, and placed in an 8 p.c. solution of trichloracetic acid (Merck's C.P.) in glass-distilled water. On arrival at the laboratory the acid was decanted and the glands ground with sand and three fresh portions of acid. The four portions were united and filtered, firstly through a Whatman No. 54 paper and then through a Jena fritted glass filter (G4 porosity). A crystal clear yellow solution was obtained.

In all but the first experiment the final volume was such that 1 ml. of extract represented 0·5 gm. of whole gland.

The protein-free extract was stored until needed without altering the acidity, which was, of course, sufficient to prevent the growth of micro-organisms. Before injection it was adjusted to pH·7 with NaOH and sterilized by addition of a few millilitres of the purest ether, which was allowed to evaporate off through sterile cotton wool overnight. The extract was then placed in sterile rubber-capped serum bottles. This simple method of sterilization was found very effective, no animals being lost through infection.

Extracts with a pH of more than about 6·5 tend to form a deposit (probably of adrenalin decomposition products) on standing. This change appears to entail no loss of activity of the solution.

Preliminary tests make it probable that the active principle cannot be extracted from the aqueous acid solution by ether. It is, thus, highly improbable that it is of a lipoidal or fatty nature. From the method of its extraction it can hardly be considered a protein. It is also very stable; one set of glands remaining in trichloracetic acid for three months yielded a highly active extract, although no special precautions were taken to exclude air or lower the temperature. These

facts remove the possibility that the luteinizing activity of our preparation is due to cortin. Although pure cortin is water soluble, as it occurs in the adrenal it is firmly attached to the cellular lipoids. Thus Grollman, Firor, and Grollman (1935) state that " . . . . attempts to extract the hormone from the glands by the use of dilute acid or alkali have been unsuccessful in our experience". The same authors also give evidence of the unstable nature of cortin.

A further study of the chemistry and possible methods of concentration of the active principle of our extracts is in hand.

THE PHYSIOLOGY OF THE EXTRACT (Geoffrey Bourne).

The association of adrenal cortical hyperplasia with such conditions as virilism in women, and sexual precocity in young male children, and the occurrence in Addison's disease of amenorrhoea in the female, and impotence and atrophy of the testes in the male, has for a very considerable time led to the conception that the adrenal gland is associated physiologically with the reproductive organs.

A review of the literature on the subject shows an indescribable confusion as to the relationship.

Migliavacca (1932 and 1933) showed that injections of cortin caused luteinization of the ovaries of young virgin female rats. Howard and Grollman (1934) do not support the suggestion that the adrenal cortex contains two hormones, one essential for life and one reacting on the reproductive system. Britton and coworkers (1933) and Casida and Hellbaum (1934) all found the adrenal cortical hormone to possess the ability to cause luteinization. Muller (1931) and Kaplan (1932) found that there was a depression of the female sex organs rather than a stimulation following injection of cortin. Martin (1932) found suppression of oestrus following adrenalectomy in the rat, and from his experiments he deduced that the cortical hormone worked through the pituitary gland.

Loos and Kittmann (1933) found that extracts of suprarenal cortex had no effect on the sex organs in either the rat or the chick.

Hicks and Matters (1935) found that in adrenalectomized rats luteinization did not appear to be accelerated by administration of cortin, and they considered that, as far as their own experiments were concerned, the adrenal cortex is not necessary for luteinization to take place.

The extract obtained by the present authors produces not only luteinization but also considerable endometrial hypertrophy.

An extract prepared as above from the adrenals of kangaroos was used. In view of the small quantity of adrenal tissue in each kangaroo, the adrenals from three specimens were used. Two of these were lactating females and the third an adult male. The animals used for testing the extracts were virgin female rats 8 to 10 weeks of age.

Injections of 0·1 ml. of the extract were given daily for eleven days. All the rats injected showed some degree of hypertrophy of the uterus and the presence of corpora lutea and blood spots in both ovaries. In two specimens the uterus was greatly enlarged.

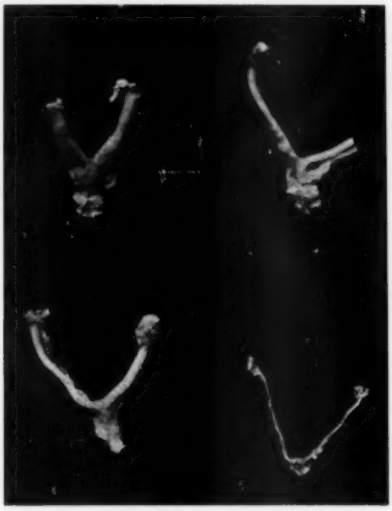

Fig. 1.

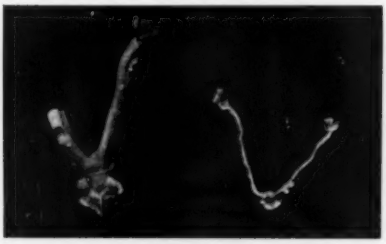

Fig. 2.

Fig. 3.

Figure 1. Top left: Uterus of virgin female rat after eleven daily injections of extract of kangaroo adrenals. Top right. Uterus of virgin female rat after six daily injections of extract of cow adrenals. (Portion of right arm removed for sectioning.) Bottom left: Uterus of virgin female rat after six daily injections of extract of pregnant cow adrenals. Bottom right: Normal uterus of virgin female rat of same age and weight.

Figure 2. Left: Uterus of virgin female rat after forty-nine daily injections of cortin (·25 ml.) (Parke Davis, "Eschatin"). Portion of left arm removed for sectioning. Right: Normal uterus of virgin rat of same weight and age.

Figure 3. Left: Normal uterus of virgin rat. Right: Uterus of virgin rat of same age and weight after six daily injections of anterior-pituitary-like hormone (Parke Davis, "Antuitrin S").

Preparations from a cow's adrenals were then made. Six daily injections of 0·1 ml. were given. The result was similar to that obtained with the extract of the kangaroo adrenals. The uterine hypertrophy was, however, only about half as great as with the injections of kangaroo adrenal extract. This was probably due to the fact that only slightly more than half the number of injections was given.

Further extracts of the adrenals of pregnant cows, administered in daily injections of 0·1 ml. and four of 0·15 ml., gave an even more marked result. Additional experiments on the occurrence of the hormone in the adrenal glands of male animals indicated the presence of the active principle in this site also.

Fig. 4.

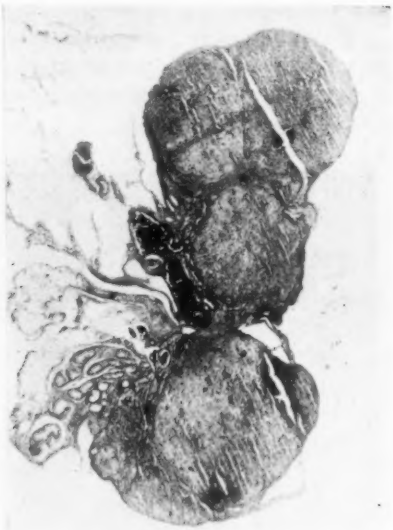

Fig. 5.

Fig. 6.

Fig. 7.

Figure 4. Ovary of virgin female rat after eleven daily injections of hormone from kangaroo adrenals. Two large corpora lutea and two follicular cysts are present.

Figure 5. Ovary of virgin female rat after six daily injections of hormone of pregnant cow adrenal. Four large corpora lutea present.

Figure 6. T.S.: Uterus of virgin female rat after eleven daily injections of hormone from kangaroo adrenals. Hypertrophy of endometrium is apparent and commencing development of glandular tissue.

Figure 7. T.S.: Uterus of virgin female rat after forty-nine daily injections of cortin (Parke Davis, "Eschatin"). Endometrial hypertrophy, but very little development of glandular tissue apparent.

It was found that injections of a preparation of the cortical hormone ("Eschatin", Parke Davis) over long periods of time had a similar effect. A dose of 0·25 ml. daily for seven weeks was used. But although considerable luteinization was observed only a relatively small degree of uterine hypertrophy occurred.

The facts at our disposal concerning the relationship of the gonads to the adrenals may be summarized as follows:

(1) The cortical hormone (cortin) is capable of causing luteinization and a slight degree of uterine hypertrophy if injected continuously over a relatively long period.

(2) In adrenalectomized animals the cortical hormone appears to have no accelerating effect upon luteinization.

(3) Aqueous extracts of the adrenal gland of the kangaroo and cow cause extensive luteinization of the ovaries and hypertrophy of the uterus. We consider this effect to be distinct from that due to cortin, and it is proposed to call the principle contained in the aqueous extract of the adrenal, "adrenoluterin".

The action of this principle appears to be very similar to that of the anterior-pituitary-like hormone obtained from pregnant urine. From the similarity of its action with the combined effects of prolan A and prolan B also there is a possibility that it may be of a dual nature.

It is possible that certain of the preparations of "cortin" used by various workers may have contained this new luteinizing factor as an adulterant, and this would perhaps be responsible for the conflicting results hitherto reported. In fact Grollman and Firor state definitely that the claims that cortin produces any functional changes in the reproductive system have been shown to be erroneous.

SUMMARY.

The preparation is described of a stable, aqueous extract of adrenal glands, which is capable of causing luteinization of the ovaries and endometrial hyperplasia when injected into immature female rats. The active principle appears to be distinct from cortin.

ACKNOWLEDGMENT.

We are greatly indebted to Mr. A. Johnson, M.R. San. I., for his invaluable assistance in obtaining supplies of fresh adrenal glands for us, and to Mr. W. J. Owen, F.R.S.E., of this Institute, for the preparation of the sections.

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THE EFFECTS OF STIMULATION OF THE ADRENAL GLAND UPON ITS CONTENT OF ASCORBIC ACID, ADRENALIN AND GLUTATHIONE

by

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(Submitted for publication, 28th November, 1935.)

In the course of investigating the possibility of producing synthesis of ascorbic acid *in vivo* by injections of mannose—positive indications of which have been reported by Guha and Ghosh (1935)—it was observed that a slight fall occurred in the average ascorbic acid content of the adrenal gland of rats following repeated injections of the mannose over a period of several hours. When the mannose was administered in a single injection five hours before killing, an increase in the ascorbic acid content of the adrenal was obtained.

It was considered that the difference might be due to the greater indirect stimulation of the adrenals in those cases where repeated injections were made.

Beznák and Hariss (1934), working with cats, observed no significant change in the ascorbic acid content of the adrenal following either stimulation, or section leading to degeneration of the splanchnic nerve, or perfusion with acetyl-choline and adrenalin. They anaesthetized their animals and made a direct comparison between the right and left adrenals, one being stimulated and the other used as a control. In our own experiments we obtained only a slight fall in the ascorbic acid content following electrical stimulation in anaesthetized rats, but a more marked fall appeared to be associated with the anaesthetization itself.

Rats of the same age and sex, and which had been fed upon a scorbutic diet, did not show a wide variation in the ascorbic acid content of their adrenal glands, providing the animals were killed rapidly by a blow on the head. A marked fall in the average content occurred, however, when animals were subjected to conditions involving adrenal stimulation prior to killing.

I. *The Effect of Ether Anaesthesia and other Methods of Stimulation upon the Ascorbic Acid Content of the Adrenal Gland.*

The methods used to produce stimulation of the adrenals were partial ether anaesthesia, the injection of acetyl-choline following a previous injection of eserine and the inducement of a direct fear reaction.

After the period of stimulation the rats were killed, the adrenals removed and dissected free from adherent tissue, and the ascorbic acid content of the combined glands determined as mg./gm. adrenal tissue. The control animals were killed by a blow on the head.

All the rats used in this series were young males of approximately the same age and weight. They were fed on a scorbutic diet for a fortnight before being used. The estimations of ascorbic acid were made by the method of Birch, Harris, and Ray (1933). Some check estimations were also made by the modification of Emmerie and van Eekelen (1934), the discrepancy between the stimulated and control animals being of the same order with both methods. The results are summarized in Table 1.

TABLE 1.

		Number of rats.	Ascorbic Acid in Adrenals. Average in mg./gm.	Range in mg./gm.
I	Controls (killed by blow on head)	9	2·74	2·38–3·10
II	Light ether anaesthesia (45 mins.)	9	1·76	1·48–1·83
III	Curari (·27 mg./100 g.) followed by ether anaesthesia (45 mins.)	5	1·80	1·54–2·01
IV	"Fear reaction"	6	2·39	1·77–2·70
V	Injection of acetyl choline (32 mg./100 g.)	3	2·50	2·31–2·78
VI	Injection of eserine (·07 mg./100 g.) followed by acetyl choline (32 mg./100 g.)	8	2·17	1·97–2·67
VII	Electrical stimulation (2 mins.) under ether anaesthesia:			
	Unstimulated	4	2·12	1·9 –2·38
	Stimulated		1·84	1·49–2·04

The most marked fall was obtained with light ether anaesthesia of 45 minutes' duration. In this procedure the animals were never completely anaesthetized, and were allowed to recover almost completely at intervals during the 45 minutes of treatment. Less pronounced falls were obtained by the injection of acetyl-choline after eserine, and by frightening the animals. In each of the three cases the difference is statistically significant, as judged by the Fisher "t" test. Acetyl-choline alone caused a slight but not significant fall.

Since Eekelen and Kooy (1935) have reported that muscular fatigue causes a profound fall in the ascorbic acid content of the liver and adrenals, it was conceivable that at least part of the reduction caused by the treatment with ether might be due to associated muscular movements. The failure of curari to obscure the effect suggests that this is not so. The fall obtained with light anaesthesia after the injection of a sub-lethal but paralysing dose of curari, was of the same order as with the ether alone.

The injection of curari without subsequent ether anaesthesia produced a very slight fall which is not statistically significant.

An attempt was made to compare the ascorbic acid content of the adrenals with and without direct electrical stimulation. Rats were rapidly anaesthetized with ether, one adrenal gland removed, and the other stimulated electrically. The

latter was then removed and the ascorbic acid content of each gland determined. The stimulated gland invariably showed a slightly lower content than the control; but the preliminary anaesthetization had caused a greater fall than the subsequent direct stimulation. If any effective form of anaesthetization could be found which did not of itself cause a loss of ascorbic acid, the fall due to direct stimulation might presumably be greater. Experiments have as yet failed to confirm this contention.

II. *The Relation of the Fall in Ascorbic Acid Content to that of Adrenalin.*

The conditions under which reductions in the average ascorbic acid content of the adrenals were observed should also have caused a discharge of adrenalin. Although it was not considered probable that there is any direct connection between the discharge of adrenalin and the disappearance of part of the ascorbic acid, experiments were conducted to determine whether the amounts of the two substances were reduced in any constant proportion under varying conditions.

For these experiments, female rats of approximately the same age were used. At the conclusion of the experimental procedure the adrenal glands were rapidly removed and extracted with 5 p.c. trichloracetic acid. The extract was then divided into portions, one being used for the ascorbic acid determination, and the other for that of adrenalin. Adrenalin was determined by the method of Baker and Marrian (1927), using a Lovibond Tintometer.

Since the amount of adrenalin lost by the adrenal glands is somewhat checked by a fairly large dose of atropine (Houssay, 1934), a comparison was made between the effects of light ether anaesthesia alone and following an injection of atropine. Although the difference is small, the ratio "ascorbic acid loss": "adrenalin loss" was greater after the injection of atropine. Conversely, the ratio after morphia injection was little more than half that obtained with ether anaesthesia, the morphia causing a greater diminution in the adrenalin content, but having less effect upon the disappearance of ascorbic acid. The terms "adrenalin loss" and "ascorbic acid loss" are used as a convenient method of describing the differences in the average amounts of these constituents between the experimental and control animals. These figures do not, of course, represent the actual amounts discharged or utilized per gm. of tissue during the period of stimulation. The results are summarized in Table 2.

TABLE 2.

		Number of rats.	Ascorbic Acid in the adrenals. Average in mg./gm.	Adrenalin in the adrenals. Average in mg./gm.	Ascorbic Acid "loss". / Adrenalin "loss".
I	Controls (killed by blow on head)	5	3·04	2·53	—
II	Light ether anaesthesia (45 mins.)	4	1·90	2·03	2·28
III	Atropin (4 mg./100 gm.) followed by ether anaesthesia (45 mins.)	6	2·09	2·20	2·88
IV	Morphia injection (3·5 mg./100 gm.)	5	2·20	1·84	1·22

III. *The Relation between the Amounts of Ascorbic Acid and Glutathione in the Adrenal Glands.*

It is well-known that the ability of the adrenal medulla to reduce silver nitrate is increased by a defence reaction. This phenomenon has been observed by us to occur following the light ether anaesthesia. Since Svirbely (1935) has shown that the silver nitrate test for ascorbic acid depends upon the relative amounts of glutathione and ascorbic acid present in the tissue or extract, experiments were undertaken to determine if the glutathione : ascorbic acid ratio in the adrenals varied as a result of the administration of ether.

For these experiments, the ascorbic acid determination was modified by the addition of 1 ml. of glacial acetic acid to the indicator solution immediately prior to titration (Harris and Ray (1933); Guha and Ghosh (1934)). By this procedure, lower and more consistent values are obtained for ascorbic acid. Glutathione was determined by the Perlezweig and Delrue (1927) modification of Tunnicliffe's method, an allowance being made for the ascorbic acid present. This procedure has been used by Phillips, Stare, and Elvehjem (1934).

For the estimation, the adrenal glands were rapidly removed, weighed, and ground up with 5 p.c. trichloracetic acid. The extract was then filtered, and the residue washed several times to make a final volume of 10 ml.; 5 ml. of this filtrate were taken for the glutathione estimation, and by adjusting the amount of indicator used, the remainder was sufficient for the ascorbic acid titration.

The estimations were made alternately on control and anaesthetized rats, only males which had been kept on a scorbutic diet for two months being used. The results are summarized in Table 3.

TABLE 3.

	Number of rats.	Glutathione in the adrenals in mg./gm. Average.	Range.	Ascorbic Acid in the adrenals in mg./gm. Average.	Range.	Glutathione / Ascorbic Acid.
Controls	8	3·61	2·24–4·99	2·04	1·87–2·28	1·77
After light ether anaesthesia (45 mins.)	8	2·04	·94–3·22	1·25	1·18–1·40	1·63

The figures for the glutathione content of the glands, in both control and experimental animals, show a wide variability; and the glutathione : ascorbic acid ratio is therefore very variable. Moreover, there is no indication of any abnormal trend in the ratio as a result of stimulation.

DISCUSSION.

From these experiments it would appear that hyperactivity of the adrenal glands is associated with loss of ascorbic acid. The greatest loss was obtained following partial anaesthetization with ether; but this may have been as much due to the fear reaction so caused as to any direct action of the anaesthetic, since deep ether anaesthesia did not produce so marked a fall. The results suggest a possible explanation of the observation (Zilva, 1935) that the use of ether anaesthesia in the intravenous injection of ascorbic acid caused an increased excretion of ascorbic acid in the urine.

The discharge or utilization of the ascorbic acid is not, however, directly proportional to the duration or intensity of the stimulus, since there is a progressive increase in the resistance to further loss. This suggests either that the gland contains an excess which is readily disposed of as a result of stimulation, or that a partial replacement from some source occurs after the initial loss. The former contention is supported by the fact that there is a slow fall in the average normal content of ascorbic acid in the adrenal gland when rats are maintained for a prolonged period upon a scorbutic diet.

The simultaneous estimations of adrenalin and ascorbic acid are of limited significance; but they suggest that there is no direct relationship between the discharge of adrenalin and the utilization of ascorbic acid. They offer, therefore, some support to the prevailing opinion (Bessey and King, 1933, and others) that the functions of ascorbic acid are related to respiration and metabolic rate rather than to a specific function in any particular tissue.

From the glutathione estimations it would seem that the increased reduction of silver nitrate by the adrenal medulla, following stimlation, cannot be ascribed to a gross change in the glutathione : ascorbic acid ratio of the whole gland. This does not, of course, preclude the possibility of a localized alteration in the medulla.

SUMMARY.

1. Light ether anaesthesia and other conditions involving stimulation of the adrenal glands cause a fall in the average ascorbic acid content of these glands.

2. The fall in ascorbic acid content bears no constant relationship to the simultaneous fall in adrenalin content.

3. The ratio of glutathione to ascorbic acid in the whole gland shows no marked variation as a result of stimulation.

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THE VASO-DEPRESSANT ACTION OF THE VENOM OF THE AUSTRALIAN COPPERHEAD *(DENISONIA SUPERBA)*

by

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(Submitted for publication 7th February, 1936.)

The Australian snake venoms have a powerful stimulant action upon plain muscle *in vitro* which closely parallels the anaphylactic response of sensitive plain muscle, having a latent period of the same order (Kellaway, 1929a). The muscle, having once reacted, is thereby desensitized to subsequent doses of venom, unless these are very large, and desensitization may also be effected without reaction by repeated treatment with very small doses. Desensitization to snake venom does not impair the response of the plain muscle to other stimuli, and, if the muscle be from a sensitized animal, it can still react to its specific anaphylactic antigen.

The intravenous injection of such Australian venoms as do not possess a coagulant principle—for example those of the copperhead *(Denisonia superba)* and death adder *(Acanthophis antarcticus)*—causes a phenomenon which resembles in some respects their action upon isolated plain muscle. Injected in moderate dosage these venoms cause a temporary fall of blood pressure, but a second dose of similar size fails to produce any effect. By the repeated administration of doses too small to cause a significant fall of blood pressure, the animal can be desensitized so that a dose of venom, which as an initial dose would have caused a profound fall of blood pressure, is without effect. In the present paper we present an analysis of this vaso-depressant action of venom.

(a) *The Effect in the Fully-Anaesthetized Animal.*

Most of our experiments have been done upon cats, but we have demonstrated the phenomenon in the monkey, dog, rabbit, and guineapig. In all these species a first intravenous injection of venom in adequate dosage causes a fall of blood pressure, and in all except the monkey a second similar dose causes no appreciable vaso-depressant effect.

For the monkey and rabbit we used ether anaesthesia, for the dog, ether, after preliminary dosage with morphine, and for the guineapig, "Numal". Cats were fully anaesthetized with ether or with Dial (0·05 gm. per kg.), and additional ether for the necessary operative procedures.

A tracheal cannula was tied in, the blood pressure was recorded by a cannula in a carotid artery, except in the guineapig, in which the recording cannula was introduced into the abdominal aorta. A record of the respirations was obtained in some experiments by connecting the side tube of the tracheal cannula to a tambour. When it was desired to study carotid sinus reflexes to anoxaemia produced by the inhalation of hydrogen, the respirations were recorded by a rubber balloon held in contact with the chest wall by a leaden cover adjusted over the animal's thorax, and connected with a recording tambour. Intravenous injections were made through cannulae tied into a saphenous or external jugular vein, and a wash-in of physiological saline from a pipette immediately followed the injection. The venom was injected in a concentration of 2 or 4 mg. per c.c. The dosage of venom in these experiments was from 0·3 to 1·0 mg. per kg.; this was sufficient to cause a striking fall of blood pressure.

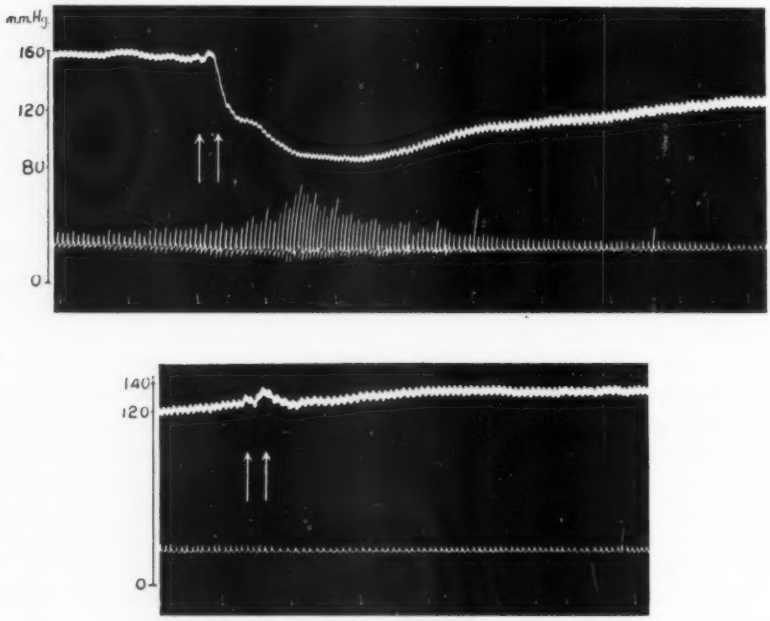

Fig. 1. Carotid blood pressure and tracheal respiratory record from a cat weighing 2·0 kg in which the vagi and one carotid sinus were intact. Between the arrows 2 mg. of copperhead venom were injected, at an interval of 5 minutes. Time in half minutes.

The fall of blood pressure which follows the first injection of venom is accompanied by evanescent changes in depth and rate of respiration. These are illustrated in Fig. 1, which is a typical result in a cat under ether anaesthesia. The fall of blood pressure is rapid, and the pressure reaches its lowest level in about a minute or a little longer, gradually recovering though seldom completely in the course of 3 or 4 minutes. In Table 1 are set out the changes in mean blood pressure

observed in a number of experiments in cats under anaesthesia following an initial intravenous injection of copperhead venom. The fall of pressure was measured at the lowest point reached, generally between one and one-and-a-half minutes of the completion of the injection.

TABLE 1.

The Changes in Mean Blood Pressure in Anaesthetized Cats following the Intravenous Injection of Copperhead Venom.

Weight in kg.	Anaesthetic.	Dose of venom in mg.	Level of mean blood pressure in mm. Hg.	Fall of blood pressure in mm. Hg.
2·97	Dial and ether	1·0	150	20
2·52	" " "	1·0	166	44
3·14	" " "	1·0	104	54
3·2	" " "	2·0	166	24
3·1	Ether	1·0	190	52
2·77	"	2·0	130	34
2·1	"	2·0	157	71
3·05	"	2·0	148	60
2·24	"	2·0	158	60

There is no obvious relationship between the original level and the observed fall of mean blood pressure. The extent of the fall of pressure is determined by unavoidable variation in the rate of injection, by individual variation and, possibly, by the depth of anaesthesia. When ether alone was used the animals were only lightly anaesthetized when the injection was made. When Dial was used the administration of ether was discontinued some time before the venom was injected.

Desensitization without striking reaction is illustrated by the following experiment:

Cat weighing 1·44 kg. under ether anaesthesia. Tracheal cannula tied in and blood pressure recorded by a cannula in the right carotid artery. A cannula was also inserted into the right saphenous vein in the thigh.

3.40 p.m. B.P. 149 mm. Hg., 0·05 mg. copperhead venom injected intravenously.
3.41 p.m. B.P. 142 mm. Hg.
3.42 p.m. B.P. 142 mm. Hg., 0·1 mg. copperhead venom injected intravenously.
3.43½ p.m. B.P. 134 mm. Hg.
3.45 p.m. B.P. 134 mm. Hg., 0·2 mg. copperhead venom injected intravenously.
3.46½ p.m. B.P. 125 mm. Hg.
3.48 p.m. B.P. 131 mm. Hg., 0·4 mg. copperhead venom injected.
3.49½ p.m. B.P. 138 mm. Hg.
3.58½ p.m. B.P. 135 mm. Hg., 2·0 mg. copperhead venom injected intravenously.
4.0 p.m. B.P. 135 mm. Hg.

The guineapig experiments were not satisfactory since the aortic pressures were low—40 to 50 mm. Hg.—very deep anaesthesia being required so that the animal would tolerate a cannula in the abdominal aorta. In five experiments in animals weighing 600–700 gm., anaesthetized with toxic dosage of Numal 0·3 to 0·4 c.c. intraperitoneally, an initial intravenous injection of 0·5 mg. of venom caused a fall of 10 to 15 mm. Hg., and second and third injections were without effect.

The experiments upon monkeys, two in number, both showed a variation from the result obtained with other species. After the pronounced fall of blood pressure following a first injection of venom, further doses continued to give small immediate but transitory vaso-depressant effects.

A monkey weighing 2·37 kg., which was anaesthetized with ether and in which the vagi were intact, received four successive intravenous injections each of 2·0 mg. of copperhead venom, at 12.11 p.m., 12.20 p.m., 12.24 p.m., and 12.27½ p.m., with the following resultant changes in mean blood pressure.

12.11 p.m. B.P. 98 mm., which fell in 1½ minutes after the injection to 60 mm. Hg.

12.20 p.m. B.P. 56 mm., fell in 15 seconds after the injection to 48 mm. Hg., and rose again as quickly to 56 mm.

12.24 p.m. B.P. 68 mm., fell in less than half a minute to 60 mm. Hg., and rose again quickly.

12.27½ p.m. B.P. 92 mm. fell to 78 mm. in half a minute, and returned within a minute to 92 mm.

This further vaso-depressant effect which could not be eliminated by desensitization may have been due to the presence of traces of a histamine-like substance in the venom. The isolated uteri of guineapigs desensitized with large doses of copperhead venom continued to give small contractions with high concentrations of the venom.

(b) *Experiments upon Decerebrate Preparations.*

That these vaso-depressant effects in the intact animal are to a large extent independent of the action of anaesthetics was shown in experiments upon decerebrate preparations in which after decerebration an interval of about an hour was allowed to elapse for the elimination of volatile anaesthetics before the venom was injected. A cat weighing 3 kg. was decerebrated, and after a suitable interval for the elimination of the anaesthetic, had a blood pressure of 200 mm. Hg. This fell to 80 mm. Hg. following the intravenous injection of 2·0 mg. of copperhead venom —a fall of 120 mm. Hg. In these experiments, as in those upon the intact cat, a second dose caused no appreciable change in the level of blood pressure.

(c) *Experiments upon Decapitate Preparations.*

These were made under deep chloroform anaesthesia after preliminary ligation of both common carotid arteries and the insertion of a tracheal cannula. We found the MacDowell (1930) clamp useful for occluding the vertebral arteries. In Table 2 are set out the effects of a primary injection of copperhead venom upon the mean blood pressure of these preparations. Fig. 2 is a typical result.

TABLE 2.

Effects of the Injection of Copperhead Venom in Decapitate Preparations.

Weight of animal in. kg.	Dose of venom in mg.	Level of mean blood pressure in mm. Hg.	Fall of blood pressure in mm. Hg.
2·32	1·0	92	26
1·39	1·0	54	24
1·95	2·0	162	120
1·25	2·0	52	22
3·0	2·0	113	48

In these experiments the extent of the observed fall of blood pressure appears to have some relation to the level of blood pressure obtaining when the observation was made. The fall of pressure from a high level when this was present was comparable to that observed in intact animals. We are evidently concerned with a

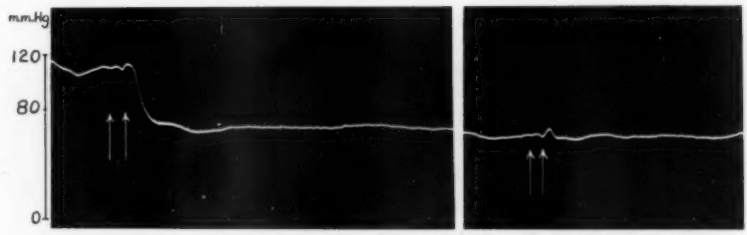

Fig. 2. Carotid blood pressure record from a decapitate preparation of a cat weighing 3·0 kg. Between the arrows, 2·0 mg. of copperhead venom were injected. An interval of 5 minutes separated the injections. Time in half minutes.

peripheral rather than with a central action of the venom, though direct action upon the vasomotor mechanism of the spinal cord is not excluded. These experiments also exclude reflexes set up by action of the venom upon the carotid and aortic sinuses, but it seemed worth while to confirm this by further experiment, since snake venoms have a paralysing action upon sensory and motor nerve endings (Kellaway and co-workers, 1932-1934).

(d) *Experiments in Animals in which the Vagi had been divided and the Carotid Sinuses excluded, denervated, or excised.*

Table 3 gives the results in four experiments in which the vagi were cut and the sinuses denervated, excluded, or excised, and of experiments in which the carotid sinuses were denervated without section of the vagi, and in which the vagi were cut but one sinus at least remained functioning. In all cases the exclusion or denervation of the sinuses was tested before injecting copperhead venom, by a brief acute anoxaemia produced by the administration of hydrogen.

TABLE 3.

Changes of Mean Blood Pressure following the Intravenous Injection of Copperhead Venom in Cats, in which the Vagi had been divided and the Carotid Sinuses denervated, excluded, or excised.

	Weight in kg.	Anaesthetic.	Dose of venom in mg.	Level of blood pressure in mm. Hg.	Fall of blood pressure in mm. Hg.
Vagi cut and sinuses denervated, excluded, or excised	2·77	Dial and ether	1·0	162	22
	2·65	„ „ „	1·0	118	32
	3·5	„ „ „	1·0	152	56
	3·2	„ „ „	1·0	116	42
Vagi intact, sinuses denervated	2·97	„ „ „	1·0	108	66
	2·22	Ether	2·0	148	78
Vagi cut, one sinus functioning	3·1	„	2·0	155	85

In the experiments in which the vagi were cut and the sinuses excluded the fall of blood pressure following a first injection of 1·0 mg. of copperhead venom ranged from 22 to 56 mm. Hg.; whereas in 3 animals of similar weight, similarly anaesthetized, but in which these structures were intact (Table 1), the fall ranged from 20 to 54 mm. This immediate fall in blood pressure cannot be accounted for even in part by action of the venom on the carotid sinuses.

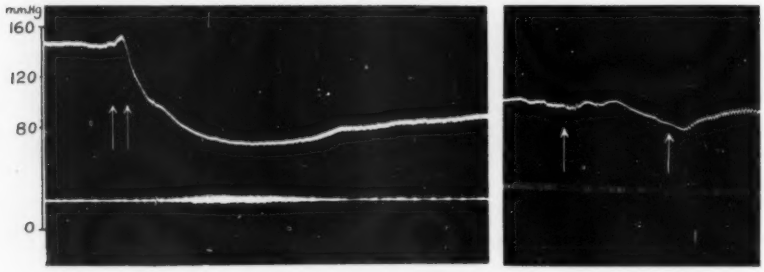

Fig. 3. Carotid blood pressure record from a cat weighing 2·2 kg. in which both sinuses had been excluded by ligature. The vagi were intact. The respirations in the left hand panel were recorded from the trachea, and in the right hand from the surface of the thorax. Between the arrows in the left hand panel 2·0 mg. of copperhead venom were injected. Those in the right hand panel indicated a period in which pure hydrogen was administered by inhalation. Time in half minutes.

Fig. 3 shows the fall of blood pressure in an animal in which both carotid sinuses were excluded, but in which the vagi were intact. The respiratory changes observed accompanying the fall of blood pressure due to the injection of venom after excision or exclusion of the sinuses in animals which no longer show any respiratory response to the inhalation of pure hydrogen, may be due to an aortic reflex mediated by the vagi, to the action of the venom on the central respiratory mechanism, or to the central effect of the fall of blood pressure itself.

In a number of experiments an attempt was made to ascertain whether, apart from any immediate effect, large doses of venom had any paralysing action upon the nerve endings of the carotid sinuses. These were wholly negative, and in no case were we able to observe any diminution in the activity of the reflexes after the administration of repeated doses of copperhead venom. This behaviour of snake venom accords with that of curari, since the fully-curarized animal also shows no impairment of the carotid sinus reflexes so long as the blood pressure is maintained at approximately the same level. Fig. 4 shows the blood pressure response to clamping the left carotid artery in a fully-curarized animal before and half an hour after the injection of repeated doses of copperhead venom to a total of 6·0 mg.

(e) *Experiments upon Curarized Animals.*

Since snake venoms have a powerful curarizing action it seemed possible that the fall of blood pressure might be related to this. A few experiments were made

to ascertain if the fully-anaesthetized and curarized animal gave a response comparable to that observed in anaesthetized but uncurarized animals. The results of these experiments, in four of which the vagi were divided, and in four of which they were intact, are set out in Table 4.

These animals were given artificial ventilation and continuous ether anaesthesia, using the Palmer apparatus with the pointer set at 2 to 2½. Curari was now given intravenously in repeated 5 mg. doses until faradic stimulation of the exposed left sciatic nerve no longer yielded any response. In some animals, further curari was given before the venom was injected.

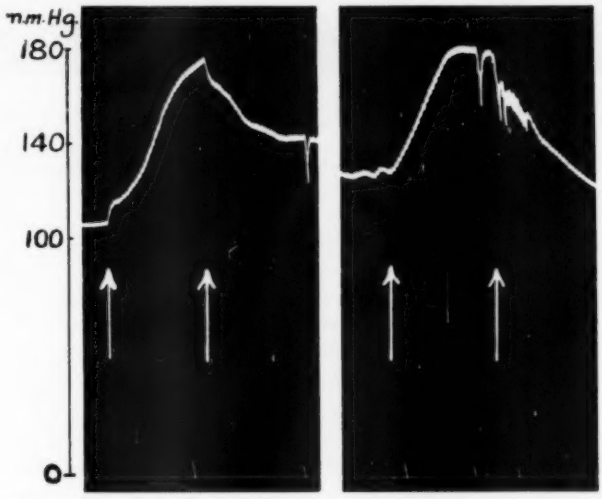

Fig. 4. Carotid sinus reflex responses to clamping a carotid artery with intact sinus in a cat weighing 3·2 kg. anaesthetized with ether and fully curarized. The left hand panel shows the response before, and the right 30 minutes after, three successive intravenous doses of 2·0 mg. of copperhead venom. Time in half minutes.

TABLE 4.

Results in Fully Curarized Animals.

Weight of cat in kg.	Dose of curari in mg.	Dose of venom in mg.	Level of mean blood pressure in mm. Hg.	Fall of blood pressure in mm. Hg.
Vagi divided				
3·8	10	2·0	144	50
3·2	15	2·0	170	27
2·95	15	2·0	126	58
3·2	20	2·0	128	20
Vagi intact				
3·7	15	2·0	116	54
2·9	22·5	2·0	98	50
4·0	35	2·0	129	63
3·24	45	2·0	108	65

In these experiments the effects of vagal section afford no evidence that central or reflex vagal effects contribute to the fall of blood pressure. They do not, however, exclude the possibility that the venom may cause a fall of blood pressure by the liberation of acetyl-choline at the vagal endings.

(f) *Experiments in Fully Atropinized Cats.*

In Table 5 are set out the changes in mean blood pressure in atropinized cats. These received repeated doses of atropine intravenously, reaching a total of 4 to 6 mg., until acetyl-choline 0·05 or 0·1 mg. injected intravenously no longer caused any fall of blood pressure. The venom was now injected, and caused a primary fall of blood pressure slightly less in extent than in animals similarly anaesthetized which had not been atropinized.

TABLE 5.

Changes in Mean Blood Pressure following the Intravenous Injection of Copperhead Venom in Fully Atropinized Cats.

Weight in kg.	Anaesthetic.	Dose of venom in mg.	Level of mean blood pressure in mm. Hg.	Fall of blood pressure.
2·69	Dial and ether	1·0	109	25
2·7	" " "	1·0	104	34
1·8	" " "	1·0	120	34
1·9	Ether	2·0	130	34
1·82	"	2·0	116	34
3·2	"	1·0	120	46

These results do not favour the view that acetyl-choline is liberated by the cytolytic action of the venom, which would also explain the contraction of the isolated uterus of the guineapig with venom, and account for the latency of this response. The observations upon isolated heart-lung preparations, now to be described, render this hypothesis untenable.

(g) *Experiments upon Isolated Heart-Lung Preparations of Cats.*

These were prepared by the technique of Knowlton and Starling (1912). The volume of blood in the system was 200 c.c. The blood flow was recorded by the Condon (1913) electro-magnetic tipper. The mean venous pressure was 5 mm. Hg. Artificial respiration was provided at a rate of 30 per minute. The injection of 2·0 mg. of copperhead venom through a hypodermic needle into the rubber connection proximal to the inflow cannula in the superior vena cava caused no striking immediate change in the heart's action, and had no appreciable effect upon the recorded arterial pressure (Fig. 5). We did four of these experiments, which yielded similar results. In the experiment figured there was a very slight decrease in the rate of flow from 35 c.c. to 33 c.c. per minute by the end of the third minute after the injection. After 20 minutes the blood commenced to darken,

and the rate of flow had fallen to 27 c.c. per minute. The heart failed from asphyxia 40 minutes after the injection. Failure was clearly due to the action of the venom upon the lung circulation, an increasing volume of lung tissue being thrown out of action. The first change observed in the lungs was a pink flush with scattered petechial haemorrhages. The end result was haemorrhagic oedema.

Fig. 5. Innominate blood pressure tracing of heart-lung preparation from a cat weighing 2·2 kg. The total volume of blood in the system was 200 c.c. At the arrow, 2 mg. of copperhead venom was injected. Flow recorded by a Condon tipper. Each signal marks the flow of 10 c.c. The right hand panel is 20 minutes later, when asphyxia was evident. Time in half minutes.

In another experiment the administration of twice the dose of venom caused a more rapid onset of these changes in the lungs, but no immediate direct effect upon the heart was observed. There was a slight change in the rate of flow from 27 c.c. to 25 c.c. per minute during the second minute. At the end of the third minute the lung changes were evidently commencing and asphyxia was beginning to affect the heart, which failed after sixteen minutes. There was extensive haemorrhagic oedema of the lungs, with many petechial haemorrhages.

It should be noted that the heart and lungs in all these experiments were subjected to a higher concentration of venom than in our experiments upon whole animals, in which the tissue damage, associated doubtless with fixation of venom, was spread over a much greater mass, 2 to 3 kg. of tissue. The operative exposure of the lungs may have aggravated the deleterious effects of the venom.

These experiments excluded a direct effect of the venom upon the heart muscle as playing any part in the immediate fall of blood pressure observed in the intact animal; furthermore, they eliminated the possibility that this might be due in part to constriction of the vessels in the lesser circulation. It seemed, however, desirable to carry out some further experiments to support this last conclusion.

(h) *The Effect of the Intravenous Injection of Copperhead Venom upon the Pressure in the Pulmonary Circulation.*

A number of experiments were made under ether anaesthesia with artificial ventilation, in which a window was made in the left side of the thorax, and the large lower branch of the left pulmonary artery was cannulized, the systemic blood pressure being at the same time recorded by a cannula in a carotid artery. Fig. 6 illustrates this type of experiment. A very slight fall in the mean pulmon-

ary pressure was observed to accompany the profound fall in the systemic pressure. It is clear, therefore, that the fall in systemic blood pressure cannot be accounted for by any diminution of inflow into the left auricle consequent upon constriction in the pulmonary circulation.

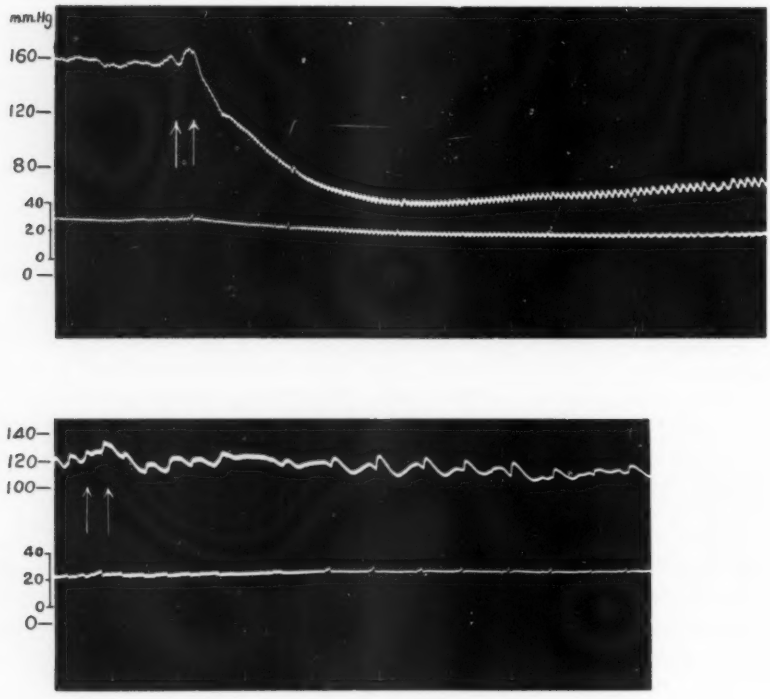

Fig. 6. Systematic and pulmonary blood pressure tracings from a cat weighing 2·65 kg. under ether anaesthesia. Between the arrows, doses of 2·0 mg. of copperhead venom were injected. The interval between the first and second dose (lower panel) was 16 minutes. Time in half minutes.

In these experiments we used a rapid and almost bloodless method of making a thoracic window. The skin was turned back by an L-shaped incision. Starting from the suprasternal notch, one limb extended to the xiphisternum and the other at right angles from the caudal end of the first incision to the posterior axillary line. The pectoral muscles were divided near their origin, and the muscular vessels twisted or tied. The eighth intercostal space was now incised and two pairs of strong bowel clamps with rubber covered blades, each with one blade within the pleural cavity, were placed in position in the long axis of the body so as to define the area of body wall to be excised. These gave perfect control of the divided intercostal vessels.

(i) *The Possible Relation of the fall of Blood Pressure to the Presence of Traces of Thrombin in the Venom.*

The intravenous injection of venoms containing thrombin results in an immediate fall of blood pressure, a fact which was emphasized by Martin's (1893, 1895, 1905) studies of the venoms of the Australian black snake *(Pseudechis porphyriacus)* and tiger snake *(Notechis scutatus)*. The venoms of the copperhead and death adder do not cause coagulation when injected intravenously, but, on the contrary, cause delay in the coagulation of shed blood, just as do minute doses of thrombin-containing venoms (Kellaway, 1929b). It seemed desirable, therefore, to exclude the possibility that the fall of blood pressure following an initial injection of these non-coagulant venoms is caused by traces of thrombin normally present in them.

The fall of blood pressure in response to the intravenous injection of venom is not diminished in a cat whose blood has been rendered incoagulable by injection of peptone. A cat weighing 3·3 kg. under ether anaesthesia which received 0·86 gm. of Witte's peptone intravenously, gave a fall of mean blood pressure of 44 mm. Hg., from 102 mm. Hg. to 58 mm. Hg., in response to a dose of 2·0 mg. of copperhead venom. In other experiments preliminary injection of hirudin also failed to impair the vascular response to venom. A cat weighing 2·07 kg., so treated, gave a fall of 42 mm. Hg., from 112 mm. Hg. to 70 mm. Hg., in response to an initial dose of 2·0 mg. of venom.

These results were not very helpful, since venom thrombins clot hirudinized and peptone plasma. However, we treated cats by repeatedly withdrawing the blood and replacing it by thoroughly whipped and filtered homologous blood. This was done both in the intact animal and in cats in which the aorta had been tied in the thorax, the abdominal viscera (except the liver and kidneys) removed and the liver excluded from circulation by tying the inferior vena cava immediately below the diaphragm.

In an intact cat weighing 3·3 kg. under ether anaesthesia, in which eight bleedings and replacements each of 30 c.c. were carried out, a dose of 2·0 mg. of copperhead venom caused a fall of 116 mm. Hg., from 172 mm. Hg. to 56 mm. Hg. A sample of blood taken immediately before the injection of venom gave only a feeble clot in 40 minutes. In a cat weighing 4·0 kg. which was eviscerated under ether anaesthesia, and in which the liver was excluded, eight successive bleedings and replacements of approximately 20 c.c. were made. The intravenous injection of 2·0 mg. of copperhead venom was now followed by a fall of 78 mm. Hg. (from a mean pressure of 120 mm. Hg. to 42 mm. Hg.). Immediately before this injection the coagulation time of the blood was one hour. After the injection of venom the blood gave only a feeble clot within 24 hours.

These results do not support the view that the fall of blood pressure is due to action of the venom upon the blood by the liberation of a depressant substance during coagulation.

(j) *Experiments upon Eviscerate Preparations.*

Experiments upon animals in which the abdominal viscera (except the liver and kidneys) were removed, and upon animals in which the aorta was ligated in

the thorax, the abdominal organs except the kidneys and liver removed and the liver excluded from circulation, gave larger responses to the injection of venom than those observed in intact cats. Some results are set out in Table 6.

TABLE 6.

The Fall of Blood Pressure following a First Injection of Venom in Eviscerate Preparations.

Weight in kg.	Anaesthetic.	Dose of venom in mg.	Level of mean blood pressure in mm. Hg.	Fall of blood pressure in mm. Hg.
Eviscerate				
3·15	Dial and ether	1·0	172	122
3·55	" " "	1·0	176	62
3·25	" " "	1·0	120	48
Eviscerate, with the aorta tied in the thorax and liver completely excluded				
3·0	Ether	2·0	154	92
2·7	"	2·0	136	116

The size of the vaso-depressant effects observed in these animals is explained by the absence of the spleen, constriction of which in the intact animal follows the injection of venom and exerts a compensating action. This was demonstrated in the following group of experiments.

(k) *Volume Changes in the Limbs, Spleen, and Bowel following the Intravenous Injection of Copperhead Venom.*

A number of experiments were made upon cats in which plethysmographic records were obtained of the volume changes, following the injection of venom, in the lower limb, in a loop of small intestine and in the spleen. In the lower limb (Fig. 7) of the curarized cat there is an increase followed by a decrease in volume which accompanies the later part of the fall, and is continued during the recovery of the blood pressure. In some experiments the initial vasodilatation was absent or marked by a fall in volume accompanying the sudden drop in blood pressure, and the response resembled that to 0·05 mg. of histamine in the figure. The changes in volume of a loop of small bowel are shown in Fig. 8; here the fall of blood pressure is constantly attended by vasodilatation. Following the injection of venom the volume of the spleen (Fig. 9) first increases and then rapidly decreases, the changes being closely similar to those following the injection of histamine. The initial vasodilatation is not constant, and in some experiments the constriction of the spleen immediately followed the injection of both histamine and of venom.

(l) *Results in Ergotoxinized Animals.*

The administration of ergotoxin in sufficient amount to paralyse the vaso-constrictors and reverse the normal response to adrenaline does not alter the re-

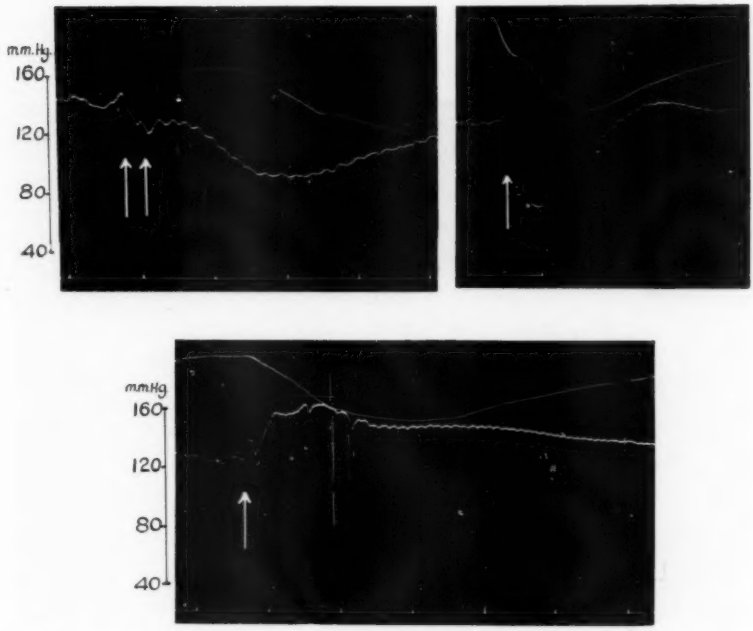

Fig. 7. Record of blood pressure and limb plethysmogram from a curarized cat weighing 3·8 kg. under ether anaesthesia, in which the vagi had been divided. Between the arrows in the left upper panel, 2·0 mg. of copperhead venom were injected intravenously. At the arrow in the right upper panel, 13 minutes later, 0·05 mg. of histamine, and at that in the lower panel, after a further interval of 4 minutes, 0·01 mg. adrenaline chloride were injected. Time in half minutes.

sponse to copperhead venom. This is a further point of resemblance to the action of venom on isolated plain muscle which also is not altered by ergotoxinization. In a cat weighing 3·5 kg. in which the vagi and sinuses were intact, 12 mg. of ergotoxin given in divided doses were necessary to cause reversal of the adrenaline effect. The intravenous injection of 2 mg. of copperhead venom caused a fall of blood pressure from 113 mm. Hg. to 84 mm. Hg. A second dose seven minutes later, when the blood pressure had risen to 158 mm. Hg., was without effect.

(m) *Experiments upon the Isolated Rabbit's Ear perfused with Oxygenated Buffered Saline.*

The results obtained with this preparation, which was set up as described in a previous paper (Kellaway and LeMessurier, 1935), except that no adrenaline was added to the perfusion fluid, gave results parallel to those obtained with the isola-

ted uterus of the virgin guineapig. A typical result is shown in Fig. 10. The initial dose of copperhead venom caused vasoconstriction, and a second dose was without effect.

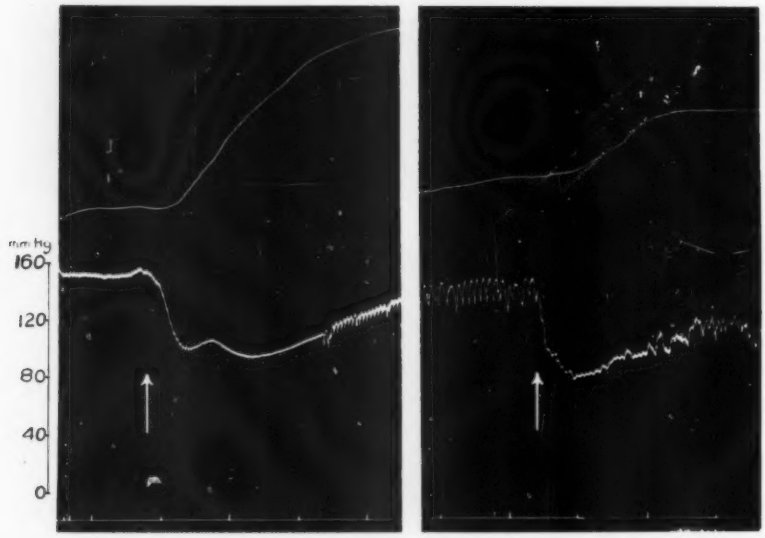

Fig. 8. Blood pressure tracing and plethysmogram of a loop of jejunum from a cat weighing 2·24 kg. under ether anaesthesia, in which the vagi were intact. At the arrow in the left hand panel 2·0 mg. of copperhead venom, and at that in the right hand panel, 7 minutes later, 0·05 mg. of histamine were injected. Time in half minutes.

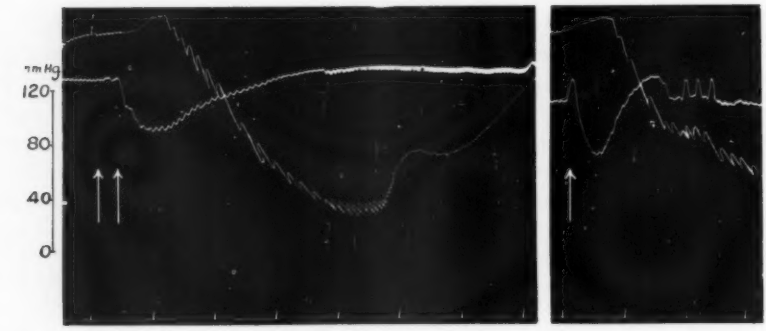

Fig. 9. Blood pressure tracing and splenic plethysmogram from a cat weighing 1·85 kg. under ether anaesthesia, in which the vagi had been cut. Between the arrows in the left hand panel, 2·0 mg. of copperhead venom, and at the arrow in the right hand panel, 7 minutes later, 0·02 mg. histamine were injected. Time in half minutes.

A number of experiments were made with cat mesentery preparations using large doses (10 to 20 mg.) of copperhead venom, both in the presence and absence of adrenaline in the perfusion fluid, but no decisive changes in the rate of flow were observed.

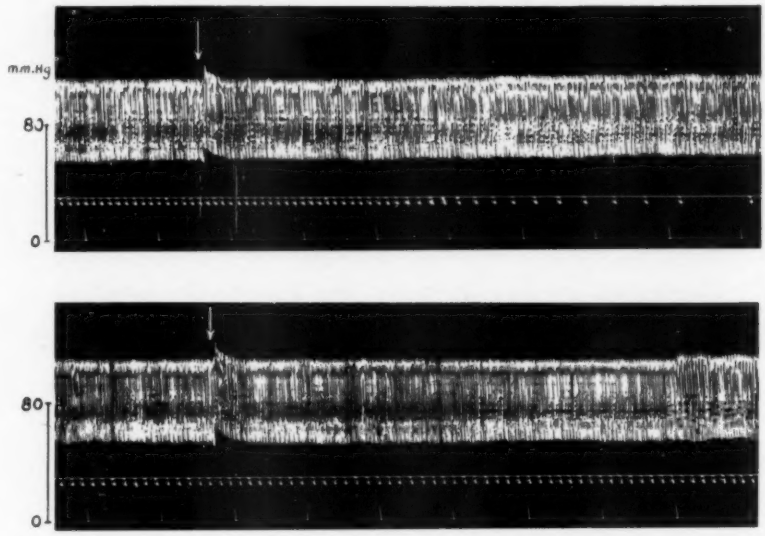

Fig. 10. Record of perfusion pressure, and rate of flow from an isolated rabbit's ear perfused with Ringer's solution. At the arrows, 0·2 mg. copperhead venom were injected immediately proximal to the perfusion cannula in the central artery of the ear. The middle tracing signals drops of the outflow. An interval of 11 minutes separated the two injections of venom. Time in half minutes.

The observations upon the isolated rabbit's ear, together with the effect of venom on the isolated guineapig uterus and the results of our plethysmograph experiments, led us to explore the possibility that histamine might be liberated by the cytolytic action of the venom, and that the vaso-depressant action under investigation might be due to this. This hypothesis was rendered somewhat unlikely by the constant occurrence of a fall of blood pressure following the initial injection of venom in the rabbit under all conditions of anaesthesia, whereas histamine in the rabbit causes a fall of blood pressure only under special conditions (Feldberg, 1927). However, the close resemblance of the stimulant action of venom on plain muscle to the anaphylactic response in sensitive plain muscle and the demonstration by Bartosh, Feldberg, and Nagel (1932), and by Dragstedt and Gebauer-Fuelnegg (1932) that anaphylactic phenomena are due to the liberation of histamine, caused us to pursue further this line of inquiry.

(n) *The Effect of Venom Injection upon the Bronchial Plain Muscle of the Guinea-pig.*

Guineapigs were deeply anaesthetized with chloroform, and a bronchial cannula was tied in The brain was pithed through the foramen magnum, and artificial ventilation was commenced. A large bored needle provided with a plunger was inserted into the right or left pleural cavity, and, after the withdrawal of the plunger, was connected to a tambour. Copperhead venom in doses of 0·5 or 1·0 mg. was then injected into a jugular vein through a fine hypodermic needle.

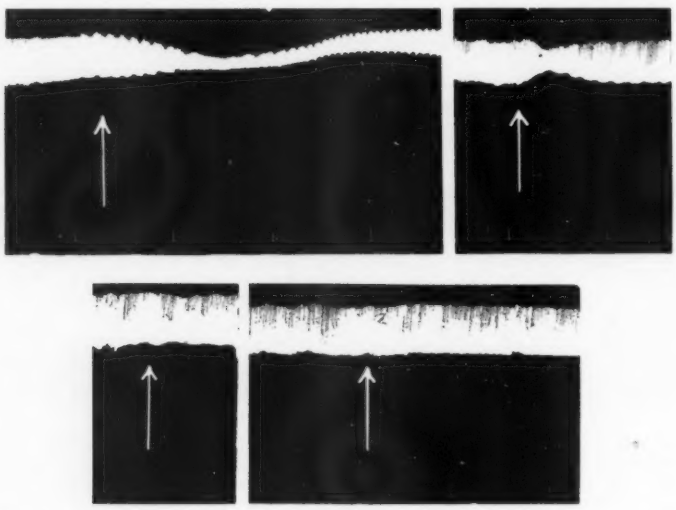

Fig. 11. Record of intrapleural pressure of a guineapig weighing 353 gm., in which the brain had been pithed under chloroform anaesthesia. In the upper left and lower right panels the arrows indicate the injection of 1·0 mg. of copperhead venom, those in the upper right and lower left panels of 0·002 mg. of histamine. The interval between the first and second panels is 1 minute, between the second and third and third and fourth, 3 minutes. Time in half minutes.

In six experiments in guineapigs of between 500 and 600 gm. weight no sign of bronchial constriction was observed. In seven experiments in guineapigs weighing between 400 and 500 gm., two showed slight transient constriction delayed until 15 to 20 seconds after the injection, and five yielded negative results. Of seven experiments in guineapigs weighing from 300 to 350 gm., three showed definite but transient contraction of the bronchial muscle delayed some seconds after the injection and not immediate as following the injection of 0·002 to 0·004 mg. of histamine. Those animals in which the response failed appeared to be less sensitive to histamine, requiring, as did the larger animals, doses of the order of 0·005 mg. to cause a transient constriction of the bronchi.

Fig. 11 shows a typical result. A second dose of copperhead venom was always

without effect. It may be noted that second doses of histamine also produced less effect than the initial dose. For this reason we usually gave the venom first. We did not do enough experiments to decide whether the injection of venom impaired the response of the animals to a subsequent dose of histamine.

These results provide a further point of resemblance between contraction of plain muscle caused by the Australian venoms and the anaphylactic response of sensitive plain muscle. Large guineapigs are much less sensitive to the broncho-constrictor action of the venom than small guineapigs. This is true also of their reaction to histamine and in acute anaphylactic shock. This decreasing sensitiveness (with increase of size) to histamine itself, to snake venom, and in sensitive animals to the anaphylactic antigen, is probably related to the increase in size of the bronchi, which in large guineapigs are much less easily obstructed by constriction of their plain muscle.

These experiments though they afford an interesting comparison with anaphylaxis do not provide proof that histamine or a histamine-like substance is liberated by the venom. Apart from the delay in response they might almost be accounted for by traces of a histamine-like substance in the venom.

This delay in response, if the response be to the venom itself and not to a contaminant, may be accounted for by the slower diffusion of an active protein constituent.

In any case the similarity to acute anaphylactic shock in the guineapig cannot be pressed too far, because guineapigs of from 200 to 250 gm. weight injected with 0·5 to 1·0 mg. of venom by a jugular vein exposed under local anaesthesia, present neither the typical asphyxial symptoms preceding death from bronchial obstruction, nor the distended lungs regularly observed in guineapigs of this weight after the intravenous injection of a lethal dose of histamine or in acute anaphylactic shock.

(o) *Titration of Perfusate from Cat's Lungs and of Lymph from the Thoracic Duct of Dogs following the Injection of Snake Venom.*

The isolated guineapig uterus was used as a test object in these experiments after desensitizing it to copperhead venom. This should make it possible to test fluids containing venom for the presence of histamine or of some substance with similar action. Such uteri suspended in a bath of 40 c.c. capacity were sensitive to doses of histamine of the order of 0·0003 mg.

In our first group of experiments we tested the perfusate from cats' lungs perfused with Ringer's solution into which copperhead venom in a dose of 1·0 mg. was injected immediately proximal to the cannula in the pulmonary artery. These experiments were wholly negative, and Fig. 12 shows a typical result. It will be observed, however, that after desensitization to the specific stimulant substance in the venom by repeated dosage with 1 mg. of venom, there still occurs a small response which may be due to the presence in the venom of traces of a histamine-like substance which is effective after desensitization.

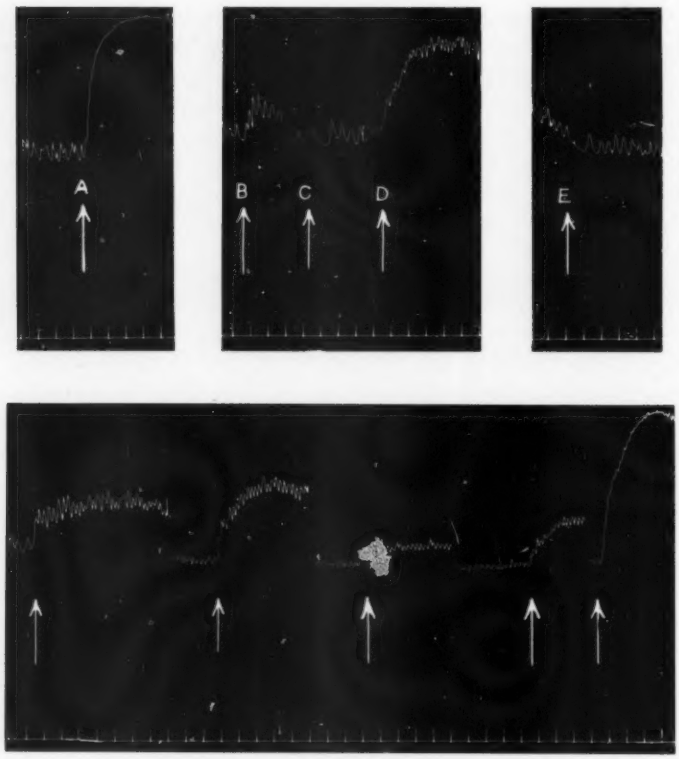

Fig. 12. Tracing from the isolated uterus of a virgin guineapig suspended in oxygenated Ringer's solution in a bath of 40 c.c. capacity. At A, 1·0 mg. of copperhead venom; at B, the fifth dose of 1·0 mg. of copperhead venom (all the preceding doses except the first having given this small contraction); at C, 10 c.c. of perfusate from a cat's lung perfused with Ringer's solution immediately following the injection of 1·0 mg. of copperhead venom, the perfusion rate having been slowed to 4·5 c.c. per minute just before the injection; and at D, without changing the Ringer's solution, 0·0006 mg. of histamine were added to the bath. At E, the second sample (10 c.c.) of perfusate from the cat's lung was tested. In the lower panel at the arrows there were tested successive doses of 0·0004 mg., 0·0005 mg., 0·0002 mg., 0·0003 mg. of histamine and, finally, of 0·0005 mg. of histamine together with 10 c.c. of perfusate obtained just before the injection of venom into the lung circulation.

The demonstration by Dragstedt and Gebauer-Fuelnegg (1932) of a histamine-like substance in the lymph in sensitive dogs following the intravenous injection of the anaphylactic antigen suggested that conclusive evidence for or against the hypothesis, that venom causes the liberation of histamine, might be obtained in this way.

Lymph was collected by cannulizing the external jugular vein and tying off the brachycephalic vein proximal and distal to the entry of the thoracic duct, and all the entering vessels except the duct. We did three of these experiments, inject-

ing 5·0 mg., 7·0 mg., and 9·7 mg. of copperhead venom intravenously into dogs weighing 13·8, 11, and 13 kg. In all of them the results of titration suggested that the lymph after the injection of venom contained some stimulant substance not present in lymph collected before this injection. Fig. 13 shows a typical example of a titration.

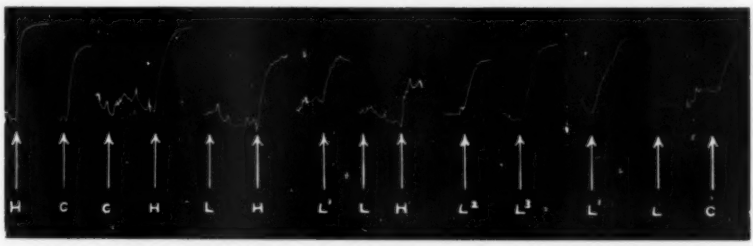

Fig. 13. Tracing of contractions of isolated uterus of a virgin guineapig used for testing lymph from the thoracic duct of a dog weighing 13kg., before and after the intravenous injection of 9·7 mg. of copperhead venom. In 45 minutes before venom 15 c.c. of lymph, in the first, second, and third periods of 10 minutes following the injection of venom, 12, 17, and 7 c.c. of lymph were collected. The last two samples of lymph failed to clot. At the arrows marked H in succession from left to right, 0·01, 0·005, 0·001, and 0·0005 mg. of histamine; at those marked L¹, L², and L³, 2 c.c. of lymph collected before the injection of copperhead venom, and in the first, second, and third ten minute intervals after injection; and at those marked C, in succession from left to right, 1·0 mg., 0·5 mg., and 0·1 mg. of copperhead venom were added to the bath. Interruptions in the tracing indicate changing of the Ringer's solution in the bath.

Unfortunately, these results cannot be regarded as decisive because the uterus in the presence of lymph becomes extremely sensitive of traces of the second stimulant substance, which are present even in small amounts of copperhead venom. An examination of other Australian venoms and fractions showed that in these also there were traces of a stimulant substance whose response was not affected by desensitization.

DISCUSSION.

The production of a vaso-depressant effect following the injection of copperhead venom presents an interesting parallel to the stimulant action of the venom upon the isolated plain muscle of the guineapig. Desensitization of the whole animal, like that of the plain muscle, can be achieved either by a single dose with resultant reaction or by a series of smaller doses without eliciting contraction in the plain muscle or significant fall of blood pressure in the intact animal. Desensitization of the whole animal with venom does not alter its response to other vasopressor or depressor substances, nor does desensitization of plain muscle affect its reaction to other chemical stimuli. Full ergotoxinization of the whole animal and of the isolated uterus does not affect their response to venom.

The vaso-depressant effect of an initial dose of venom occurs in a considerable range of species; it is not increased by anaesthesia, nor is it contributed to reflexly

by action of the venom on the tensor- or chemo-receptors of the carotid sinuses. It is almost wholly due to peripheral and not central action. Our experiments upon the isolated heart-lung preparation exclude a cardio-depressant effect of the venom as the cause of the phenomenon, which also cannot be explained by any constriction in the lesser circulation with resultant lessened cardiac intake. The initial fall of blood pressure is not related to the curari-like action of the venom, nor is it due to any by-product of coagulative changes in the blood from traces of thrombin in the venom.

Conceivably, the vaso-depressant action of the venom and its stimulant action on plain muscle might be due to the liberation of histamine by the cytolytic action of the venom. This hypothesis is attractive because it fits well with the latency observed in the contraction caused in smooth muscle by venom, and with its resemblance to the anaphylactic response of the uterus of sensitized guineapigs, with the rather feeble and delayed broncho-constriction observed in small pithed guineapigs, and also with the constriction of the vessels of the isolated rabbit's ear. However, the occurrence of a strong vaso-depressant effect of venom in the rabbit deeply anaesthetized with Dial and ether, and our failure to detect histamine in the perfusate from a cat's lung following the exhibition of venom, using as a test object the isolated guineapig uterus desensitized with snake venom, speak against this hypothesis. The apparently favourable results obtained in tests for stimulant substances in lymph from the thoracic duct of dogs after injection of the venom are rendered suspect by the presence in the venom of traces of a stimulant substance, the response to which persists after desensitization to its main stimulant action. Furthermore, the congestion of the dog's liver caused by venom is slight in comparison with that produced by a lethal dose of histamine or in acute anaphylactic death.

The evidence is, therefore, insufficient to sustain the view that these various effects produced by venom are mediated by the liberation of histamine from the tissues, and it appears more likely that they are due to the direct action of venom upon plain muscle.

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